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Flavonoids Can Protect Maize DNA from the Induction of Ultraviolet Radiation Damage[1]

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Diverse flavonoid compounds are widely distributed in angiosperm families. Flavonoids absorb radiation in the ultraviolet (UV) region of the spectrum, and it has been proposed that these compounds function as UV filters. We demonstrate that the DNA in *Zea mays* plants that contain flavonoids (primarily anthocyanins) is protected from the induction of damage caused by UV radiation relative to the DNA in plants that are genetically deficient in these compounds. DNA damage was measured with a sensitive and simple assay using individual monoclonal antibodies, one specific for cyclobutane pyrimidine dimer damage and the other specific for pyrimidine(6,4)pyrimidone damage.

Maintaining the integrity of DNA despite damage induced by UV radiation is of critical importance to all organisms that live in sunlight. Two basic strategies are available to ameliorate DNA damage: DNA repair and shielding to minimize DNA damage. Solar radiation contains wavelengths essential for photosynthesis as well as wavelengths that can damage plant DNA. Therefore, plants may have evolved particularly active DNA protection and repair mechanisms; however, to date relatively little has been established about these processes or the extent of irradiation-induced DNA damage in plants (Stapleton, 1992).

UV radiation is generally classified into three wavelength ranges: UV-A (320–390 nm), UV-B (280–320 nm), and UV-C (less than 280 nm). The absorption spectrum of DNA includes wavelengths from 240 to 310 nm; however, the level of solar UV that reaches the surface of the earth is high in the UV-A region of the spectrum, decreases sharply in the UV-B range, and drops to nearly zero by 290 nm (Robberecht, 1989). Many studies of the effects of UV radiation have employed germicidal lamps with peak output at 254 nm, in the UV-C region, although this wavelength is not present in sunlight at the earth's surface.

Measurements in situ demonstrate that the epidermis of plants absorbs 90 to 99% of incoming UV radiation; flavonoid compounds and cuticular waxes are most likely to be the principal agents of UV absorption (Robberecht and Caldwell, 1978; Caldwell et al., 1983). Long-term damaging effects of UV radiation on plants include growth inhibition and morphological alterations; these types of damage can be observed after a few days of UV-B radiation (Tevini and Teramura, 1989). UV-induced photosynthesis defects can be observed within a few hours (Tevini et al., 1991). Most early experiments used stress treatments (high light, nutrient deficiency, or UV) to induce the accumulation of flavonoids, and then measured UV damage to DNA, photosynthesis, or growth after substantial flavonoid accumulation (Mirecki and Teramura, 1984; Murali and Teramura, 1985; Takahashi et al., 1991; Tevini et al., 1991). In this protocol, however, the stress treatments may induce other responses, in addition to flavonoid accumulation, that affect the perception or response to UV. Mutants that have a specific defect in flavonoid biosynthesis provide the best experimental material for dissection of flavonoid function. Li et al. (1993) used mutants of *Arabidopsis thaliana* defective in early steps of flavonoid synthesis to demonstrate the protective effect of flavonoids. Their experiments showed that flavonoids protect *A. thaliana* from UV-induced growth inhibition measured 20 to 30 d after treatment. The specific defect or defects responsible for growth reduction in *A. thaliana* by UV-B has not been determined.

UV radiation can damage DNA as well as other cellular components, such as proteins (Grossweiner and Smith, 1989). Among the immediate plant responses to UV is a lower photosynthetic efficiency, caused by damage to proteins (Melis et al., 1992; Wilson and Greenberg, 1993). Subsequent consequences of UV damage, including induction of mutations, are more likely to involve DNA damage. UV-induced DNA damage has been extensively studied in microorganisms and mammalian cells; from these studies we know that UV-induced DNA damage must be repaired or bypassed to allow DNA replication and transcription and that DNA damage may also trigger growth arrest and changes in gene expression (Kornberg and Baker, 1992). The best-studied type of UV-induced damage to DNA is the CPD, the result of dimerization of adjacent pyrimidines on the same strand of DNA (Cadet et al., 1992). CPD constitutes 50 to 80% of the UV-specific DNA photoproducts, with most of the rest being pyrimidine(6,4)pyrimidone (Mitchell and Nairn, 1989).

CPD production and removal (repair) have been measured in only a few plant species (McLennan, 1987). For example, an action spectrum of CPD formation in alfalfa cotyledons has shown that CPDs are created by all wavelengths of UV radiation, as has been observed in human skin and in bac-

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Abbreviations: C-C, cytosine dimer; CPD, cyclobutane pyrimidine dimer; C-T, cytosine-thymine dimer; ESS, enzyme-sensitive site; TEV, T4 endonuclease V; T-T, thymine dimer.

teriophage T4 DNA (Quaite et al., 1992). Shortwave (primarily 254 nm) radiation has been used to induce CPD in *A. thaliana* (Pang and Hays, 1991). Removal of this radiation-induced damage was measured in the light and in the dark. Measurement of the removal rate in the light gives the total recovery rate; measurement of the removal rate in the dark subtracts the contribution of photolyase. Photolyase is an enzyme that specifically monomerizes CPD; this enzyme utilizes light (in the 375- to 450-nm region of the spectrum) to provide the energy for monomerization (Sancar and Sancar, 1988). Pang and Hayes (1991) found that in *Arabidopsis*, CPD removal was 10-fold faster in the light than in the dark, indicating that photolyase is a major contributor to recovery from UV irradiation. Furthermore, removal was rapid; half the CPDs were removed in about 1 h in the light. UV-induced DNA damage and repair have also been examined in maize pollen; again, both photoreactivation and dark repair were found (Ikenaga et al., 1974; Jackson, 1987).

To analyze the specific role of flavonoids in shielding DNA from UV, we have measured UV-induced DNA damage in *Zea mays* lines with the *B* and *Pl* regulatory alleles that allow expression of all flavonoid compounds and in near-isogenic *b, pl* lines specificially defective in flavonoid accumulation. The B and Pl genes control the expression of chalcone synthase, the first enzyme in the flavonoid biosynthetic pathway (Dooner, 1983). Thus, green *b, pl* lines have no flavonoids. Maize tissues that allow expression of all flavonoids contain primarily purple anthocyanins (Styles and Ceska, 1972; Stafford, 1990); the anthocyanins in maize are a mixture of at least five different anthocyanins with partially overlapping and broad UV-absorption properties (Harborne and Grayer, 1988). We refer to *B, Pl* permissive lines as purple and *b, pl* lines with no flavonoid synthesis as green, and we have investigated the ability of flavonoids to protect maize DNA in situ from UV-induced DNA damage.

A commonly used method for determining the content of CPD in DNA utilizes enzymes such as endonuclease V (encoded by bacteriophage T4) to nick the DNA backbone at CPD sites. The nicked DNA is then denatured and size-fractionated by electrophoresis, velocity sedimentation, or alkaline elution. The size of the single strands is used to calculate the frequency of CPD detected by TEV (Achey et al., 1983). The sensitivity of this assay is limited by the size of the DNA employed; about 3 CPD/megabase pair are detectable in DNA by electrophoresis in unidirectional pulsed-field alkaline gels (Freeman et al., 1986). For optimal sensitivity this assay requires very high mol wt DNA (>100 kb). To overcome this constraint we designed a simple, sensitive assay for CPD and for 6,4 photoproducts that uses small amounts of DNA of any size (Stapleton et al., 1993). Our assay uses monoclonal antibodies that specifically recognize these DNA photoproducts (Mori et al., 1991) and measures antibody binding by enhanced chemiluminescence (Roswell and White, 1978). We can use as little as 100 ng of DNA to detect as few as 4×10^7 CPD (0.2 CPD/megabase pair); the procedure uses commonly available equipment and takes 3 to 4 d for each experimental cycle.

MATERIALS AND METHODS

Plant Material

Zea mays line K8 is an inbred line in the W23 background; it contains all the structural genes required to produce anthocyanins and the regulatory alleles *B* and *Pl*, which confer anthocyanin expression on nearly every tissue of the plant. For green tissue we used line J113, which is isogenic to K8 except for recessive alleles of three regulatory genes (*r-g, b,* and *pl*) required for flavonoid accumulation. Maize plants with these recessive alleles have no detectable anthocyanins (Styles and Ceska, 1972). Plants used for sheath irradiations were grown in a greenhouse with supplemental visible lighting to 50% of summer sun. For experiments with purified maize DNA, the line ASB39 was used as a DNA source. ASB39 carries the *bz2* mutation in a background of 75% W23, 25% K55.

Flavonoid Extraction

Purple and green husk tissue (5 g fresh weight) was frozen in liquid nitrogen and ground to a powder with a mortar and pestle. The powder was extracted once for 16 h with 10 mL of acidic methanol (1% HCl in methanol) followed by eight 1-h extractions each with 5 mL of acidic methanol. The extracts were combined and then diluted 1:3000 in acidic methanol for measurement of *A*. The green extract was used as a reference.

UV Irradiation

As a UV-C source one GL-15 germicidal lamp (incident irradiance of 30 J m^{-2} s^{-1}) was positioned 12 cm above the sample to irradiate DNA. Four GL-15 lamps 12 cm from the sample (two above and two below) were used for sheath tissue irradiations. UV-B was provided by a UV-wavelength solar simulator of two Westinghouse FS-20 bulbs with two sheets of Kodacel (Kodak) and samples were placed 20 cm from the light source (Sisson and Caldwell, 1975). The Kodacel was changed after every 20 h of lamp use to avoid photochemical degradation of the filter. Tissue samples were irradiated for equal times on each side, and the total amount of incident UV applied was considered the UV dose. The output of the UV-B source (Fig. 1) was measured with an Optronics model 752 spectroradiometer (Optronics Laboratories, Orlando, FL) that was calibrated before each use. A shortwave meter, model J225 (UV Products, San Gabriel, CA), was used to measure UV-C output.

DNA Isolation

Plasmid DNA was prepared by alkaline lysis (Sambrook et al., 1989), purified using a Qiagen plasmid kit (Qiagen Corp., Chatsworth, CA), then linearized with *Not*I. The 15.6-kb plasmid pUC21::gHFSal4.2, with a 13-kb mouse DNA insert, was a gift from J. Hershberger; this plasmid has no homology to sequences in the maize genome by Southern analysis using our standard, stringent conditions (Walbot and Warren, 1988). Maize genomic DNA was prepared from immature

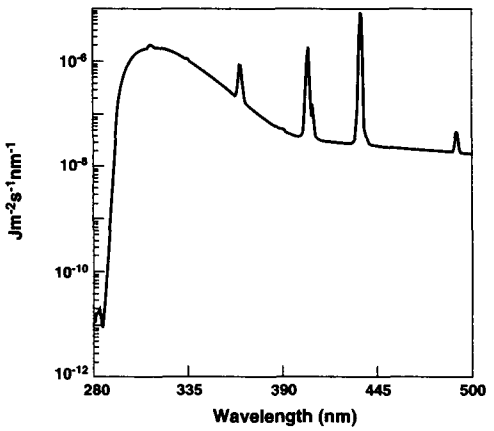

Figure 1. Irradiance of the UV-B solar simulator. Output of the solar simulator was measured with an Optronics model 752 spectroradiometer. Irradiance was measured every 1 nm from 280 to 500 nm.

ears as described previously (Rivin et al., 1982) except that only one CsCl purification was used. Sheath DNA was prepared by powdering tissue samples in liquid nitrogen, then adding 0.6 mL of final lysis buffer (0.35 M NaCl, 1 mM Tris-HCl, pH 7.5, 50 mM EDTA, 7 M urea, 2% sarkosyl). The resulting slurry was incubated with shaking at 37°C for 10 min, then 0.6 mL of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the solution was vortexed for 30 s. The mixture was then decanted into a 1.5-mL microfuge tube and spun in a microfuge at 12,000 rpm for 5 min at room temperature. The aqueous layer was removed to a new tube and mixed with one-tenth volume of 3 M sodium acetate, pH 5.2, and 3 volumes of ethanol. Tubes were inverted to mix and the contents were spun in a microfuge for 1 min. After removing the supernatant, the pellet was resuspended in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and stored at −20°C until use. Because anthocyanin carryover in the DNA minipreps inhibited the detection of chemiluminescence, samples were checked for carryover by measuring A_{530} and by visually checking for brown pigment in the DNA-containing area of membranes after slot blotting. However, samples of K8 purple sheath tissue from field-grown plants always had anthocyanin carryover. Thus, the DNA samples from field-grown plants were further purified over CsCl gradients. Gradients were prepared without ethidium bromide, fractions were collected, and a portion of each fraction was electrophoresed to locate the genomic DNA. The purified DNA was then slot blotted to assay UV damage.

TEV Treatment

TEV lot 25, a preparation capable of generating 5×10^{11} to 8×10^{11} nicks min^{-1} μL^{-1} in UV-irradiated DNA, was kindly supplied by Dr. P.C. Hanawalt. The enzyme was stored in a buffer of 100 mM Tris-HCl, pH 8.0, 1 M NaCl, 100 mM EDTA, 10% PEG. DNA was treated for 15 min at 37°C in 10 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, 1 mg/mL BSA, plus 2 μL of the TEV enzyme. Storage buffer without TEV was added to the DNA samples that were not treated with TEV.

Alkaline Gel Electrophoresis

DNA samples were denatured by adding alkaline loading dye (Sambrook et al., 1989) and then were electrophoresed through 0.6% alkaline agarose gels with a running buffer composed of 30 mM NaOH, 1 mM EDTA. Gels were electrophoresed at 20 to 40 V overnight with buffer recirculation, rinsed in distilled water, neutralized in 0.5 M Tris-HCl, pH 7.8, 1.5 M NaCl for 40 min, stained in 50 mg/mL ethidium bromide, destained in distilled water, and photographed with Polaroid type 55 film. Negatives were traced in a model GS300 scanning densitometer (Hoefer, San Francisco, CA). For each DNA sample, there were two lanes, one for the sample treated with TEV and one for the sample without TEV. Each lane was scanned for the same distance (as measured from the wells) and the area under the curve was calculated. A line was positioned on the graph so that the area was divided into equal halves (i.e. half the signal below the line and half above the line). The distance of this 50% line from the wells was recorded. The distance of the 50% line in each plus-TEV lane was divided by the distance of the 50% line in the minus-TEV lane and multiplied by 100 to give the percent damage for individual samples after UV treatment. Under our conditions, area is proportional to the molecular mass of the DNA on the gel. The calculation of the distance of the 50% line from the well gives a qualitative evaluation of DNA damage levels rather than the exact number of CPDs in each sample.

Irradiation of Plasmid DNA Controls

Twenty micrograms of linear plasmid DNA (pUC21::gHFSal4.2 digested with *Not*I) were irradiated with a germicidal lamp of output 0.75 J m^{-2} s^{-1} to a final UV-C dose of 5, 10, or 20 J/m^2. The number of CPDs in each plasmid preparation was measured by TEV analysis as described (Bohr and Okumoto, 1988). For example, in 40 ng of plasmid irradiated with 5 J/m^2, we found that there were 0.742 ESS/fragment and thus 3.46×10^9 ESS in 40 ng; this corresponds to 3.46×10^9 CPDs in 40 ng. The irradiated plasmids containing a known number of CPDs were used as internal standards in the antibody-detection experiments.

Assay of Antibody Binding to Irradiated DNA

The protocol for measurement of CPD has been described in detail (Stapleton et al., 1993). Monoclonal antibodies specific to CPDs (TDM-2) and pyrimidine(6,4)pyrimidones (64M-2) were kindly supplied by Dr. Toshio Mori (Mori et al., 1991). Sheath sections of 0.3 to 0.8 g were harvested from two to four maize plants after 6 to 8 weeks, and green and purple samples were irradiated simultaneously. Tissues were immersed in liquid nitrogen immediately after irradiation and stored in the dark at −80°C. DNA extracted from UV-irradiated plant tissues was denatured in 0.3 M NaOH for 10 min, neutralized by addition of 4 M ammonium acetate

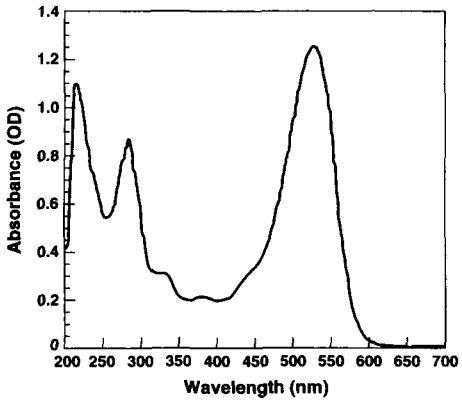

Figure 2. Absorption spectrum of extracted flavonoids. Purple and green husk tissue (5 g fresh weight) was frozen in liquid nitrogen and ground to a powder in a mortar and pestle. The powder was extracted with acidic methanol as described in "Materials and Methods." The green extract was used as a reference.

to 1 M final concentration, and slot blotted onto Hybond nylon membrane (Amersham) in quadruplicate. Plasmid DNA of known concentration with a known number of CPDs (determined by TEV assay) was included on each blot; plasmid DNA was denatured, neutralized, and slot blotted as described above for tissue DNA samples.

Antibody binding and detection with enhanced chemiluminescence were essentially as described in the manufacturer's protocol (Amersham). The blot was blocked with 5% dried milk in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween 20) for 1 h at room temperature or overnight at 4°C. The blot was then washed and reacted with TDM-2 (1:2000 in TBS-T) or 64M-2 (1:1000 in TBS-T) for 1 h with agitation. Unbound antibody was washed away, and secondary antibody (Sigma) conjugated to horseradish peroxidase (1:3000) was incubated with the blot for 1 h. The blot was washed, the detection reagents were added, and the blot was exposed to X-Omat autoradiography film (Kodak). Several exposures were performed so that the signal from each slot was in the linear range of sensitivity of the film.

Determination of DNA Concentration on Blots

The amount of DNA in each slot of each blot was determined by hybridization analysis (Sambrook et al., 1989). Blots were stripped of protein by incubation in boiling 0.1% SDS for 5 min before hybridization analysis, and 2 μg of genomic DNA plus 1 ng of pUC21::gHFSal4.2 *Not*I was labeled with ^{32}P using a random-primed reaction (United States Biochemical, Cleveland, OH) and hybridized to the blot. Blots were exposed to film for sufficient time to generate a signal that was in the linear range of the film.

Densitometry of Antibody and Hybridization Films

Films were traced in a Quick Scan R & D densitometer (Helena Laboratories, Beaumont, TX), and the peak height of the antibody signal was adjusted for DNA concentration by normalizing the DNA hybridization signal and dividing the antibody signal peak height by this value. The adjusted peak heights were converted to the number of CPDs using the peak height determined from the plasmid control, with a known number of CPDs, on the same blot.

The means of the four identical samples from each blot were plotted, and a permutation test (Edgington, 1988) was used to compare two sets of four data points to each other. The permutation test compares all the possible means of two sets of numbers to generate a two-sided P value. For example, comparison of 1, 2, 3, 4 to 5, 6, 7, 8 generates a P value of 0.0285, which is significantly different at the 95% level. Comparison of 1, 2, 3 to 4, 5, 6 gives a P value of 0.1, which is significant at the 90% level.

The entire experiment (starting with new plants) was repeated with similar results. The results were not combined because plants harvested at different times had different background CPD levels.

RESULTS

We wished to test the hypothesis that flavonoids offer protection from UV-induced DNA damage in vivo. We used sheath tissue from near-isogenic purple and green maize plants; purple sheath tissue (*B, Pl*) contains high levels of flavonoids (primarily anthocyanins), whereas green tissue (*r-g, b, pl*) contains no detectable anthocyanins (Styles and Ceska, 1972; Stafford, 1990). The absorption spectrum of the flavonoids extracted from purple maize tissue is shown in Figure 2. For comparison, the anthocyanin concentration in the maize tissues we used in our experiments is approximately the concentration found in red cabbage.

First, we used TEV analysis to measure the number of CPDs induced by UV-C irradiation. Dose-response experiments were performed on sheath tissues (data not shown), and doses that produced a substantial level of DNA damage were chosen for the comparison of green and purple tissue. During these long UV-C irradiation periods, the temperature inside the UV-C irradiation chamber increased from 25 to 28°C after 500 s (6000 J/m^2). In the dose-response experiments we noticed that the amount of DNA recovered from tissue decreased with increased irradiation times. This is probably the result of cross-linking of DNA to proteins in the outer layers of the tissue (i.e. in the epidermis), which is exposed to very high levels of UV. Thus, to quantify shielding

Table I. *TEV assay for CPDs*

As described in "Materials and Methods," the extent of damage was determined by comparing sample pairs (with or without TEV) from green and purple sheath sections at each UV dose. The midpoint of the DNA size distribution from the +TEV sample in each pair was divided by the midpoint of the −TEV sample. This number was multiplied by 100 to determine the percent undamaged DNA.

	UV-C Dose (J/m^2)					
	0		1200		6000	
Anthocyanin content	Green	Purple	Green	Purple	Green	Purple
Undamaged DNA (%)	92	100	75	109	10	54

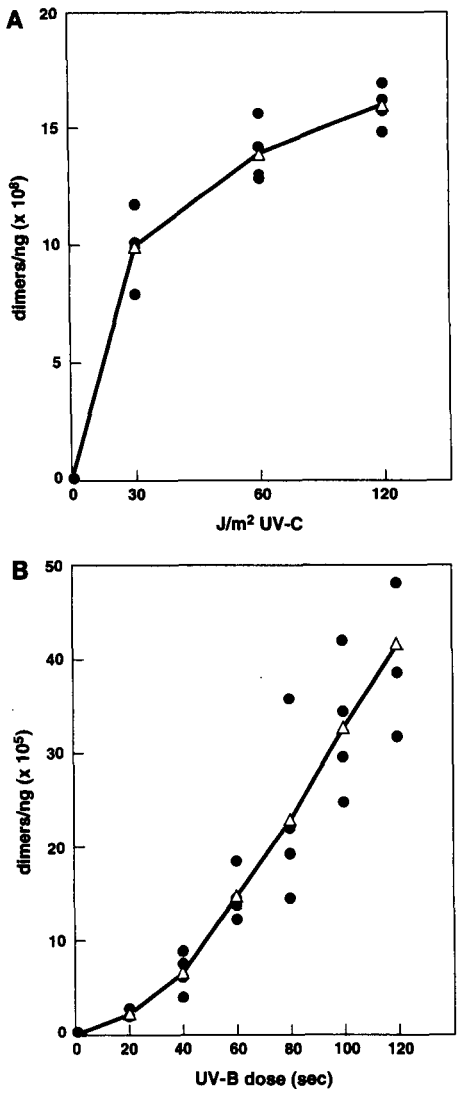

Figure 3. Dose response of purified DNA irradiated with UV radiation. ●, Individual measurements; Δ, average. A, Fifteen nanograms of genomic DNA were irradiated for 0, 1, 2, or 4 s (0, 30, 60, or 120 J/m²) with UV-C. The DNA were reacted with the TDM-2 antibody and the antibody signal was detected with chemiluminescence as described in "Materials and Methods." The amount of DNA in the antibody reactions was normalized by hybridization with ^{32}P-labeled DNA. The chemiluminescence signal and the hybridization signal were quantified by densitometry, and the antibody signal was adjusted to reflect the actual amount of DNA on the blot. The signal from 9 ng of 20 J/m² plasmid control was used to determine the number of CPDs in the genomic samples. In the exposures that were used for densitometry the signal from 0 J/m² UV-C was undetectable. B, Ninety nanograms of genomic DNA were irradiated with UV-B (output of UV-B source shown in Fig. 1) for 0, 20, 40, 60, 80, 100, and 120 s.The DNA was reacted with the TDM-2 antibody and the antibody signal was detected with chemiluminescence as described in "Materials and Methods." The amount of DNA in the antibody reactions was normalized by hybridization with ^{32}P-labeled DNA. The chemiluminescence signal and the hybridization signal were quantified by densitometry, and the antibody signal was adjusted to reflect the actual amount of DNA on the blot. The signal from 4.5 ng of 5 J/m² plasmid control was used to determine the number of CPDs in the genomic samples. In the exposures that were used for densitometry the signal from 0 J/m² UV-C was undetectable.

we compared CPD content in samples from green or purple tissue at each dose rather than to the unirradiated samples.

The results of the TEV experiments are shown in Table I. Before irradiation there was little difference in the steady-state level of CPDs in green and purple tissues. After 1200 J/m² of UV-C there was less damage in the purple tissue than in the green tissue. After 6000 J/m² of UV-C the protective effect of the anthocyanins was more pronounced (10% undamaged DNA left in the green sample and 54% undamaged DNA left in purple samples).

It was necessary to use very high UV-C doses to measure damage with the TEV assay (damage was visible in the purple sample only after 6000 J/m² UV-C), and we were unable to detect DNA damage in dose-response experiments using the lower-fluence, solar-type UV-B source. Thus, we turned to a more sensitive assay for UV-induced DNA damage. This assay uses enhanced chemiluminescent detection of antibody bound specifically to damaged DNA bases. Comparison of the signal in treated samples with the signal from plasmid DNA containing a known number of CPDs allowed us to determine the number of CPDs in the irradiated plant DNA. Figure 3A shows the results of experiments in which we irradiated purified genomic DNA with UV-C and measured the number of detectable CPDs using the TDM-2 monoclonal antibody. At 30 J/m² UV-C, 7.5×10^9 CPDs were generated in the 15 ng of plant DNA.

Dose-response curves from purified DNA irradiated with UV-B are shown in Figure 3B. UV-B doses are given in seconds. The UV-B output of our lamp system was comparable to the UV-B output of sunlight as measured at the surface of the earth (Sisson and Caldwell, 1975). For UV irradiations of mammalian cells, doses are often weighted using the action spectrum for erythema induction (Parrish et al., 1982) to allow facile comparisons of different UV sources. However, there is no generally accepted action spectrum for plants that is analogous to the erythema action spectrum in mammals (Coohill, 1989; Quaite et al., 1992; Middleton and Teramura, 1993). Thus, we give the irradiance of our UV-B source (Fig. 1) and then give doses in seconds of this output. Figure 1 shows that the energy from the UV-B solar simulator is spread out over wavelengths from 280 to 500 nm. Since there is no generally accepted action spectrum (and thus no accepted weighing function), we cannot compare such broad-band radiation to narrow-band (primary output 253.7 nm) UV-C radiation.

It is known that UV-B creates proportionally more T-T than C-T or C-C dimers compared to UV-C (Mitchell et al., 1992). However, because the precise specificity of the TDM-2 antibody for the different types of CPD is not yet known, we could not assume that the antibody binds to the same number of CPD in the UV-C-irradiated plasmid control and in UV-B-irradiated samples. Thus, we compared plasmid irradiated with our UV-B source with UV-C-treated plasmid; when DNA with the same number of CPDs (as assayed by

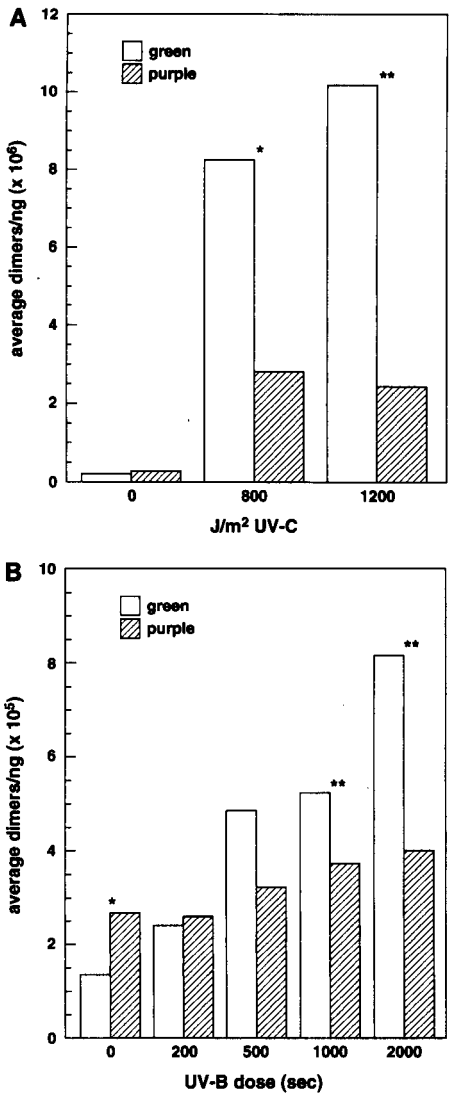

Figure 4. CPD DNA damage in green (without anthocyanins) and purple (with anthocyanins) sheath tissue irradiated with UV radiation. A, Freshly excised sheath tissue was irradiated for 0, 5, or 10 s (0, 600, or 1200 J/m^2) with UV-C. The tissue was immediately frozen in liquid nitrogen; DNA was prepared as described in "Materials and Methods." One hundred nanograms of genomic DNA were reacted with the TDM-2 antibody and the antibody signal was detected with chemiluminescence as described in "Materials and Methods." The amount of DNA in the antibody reactions was normalized by hybridization with ^{32}P-labeled DNA. The chemiluminescence and hybridization signals were quantified by densitometry, and the antibody signal was adjusted to reflect the actual amount of DNA on the blot. The signal from 3 ng of 20 J/m^2 plasmid control was used to determine the number of dimers in the genomic samples. The permutation test comparing 5-s green samples with purple samples gave P = 0.057; comparison of green and purple samples after 10 s gave P = 0.028. One asterisk denotes a P value of ≤0.1; two asterisks denote a P value of ≤0.05. B, Freshly excised green and purple sheath tissue was irradiated for 0, 200, 500, 1000, or 2000 s per side with UV-B (irradiance of UV-B source shown in Fig. 1). The tissue was immediately frozen in liquid nitrogen, and DNA was prepared as described in "Materials and Methods." Four hundred nanograms of genomic DNA were reacted with the TDM-2 antibody and the antibody signal was detected with chemiluminescence as described in "Materials and Methods." The amount of DNA in the antibody reactions was normalized by hybridization with ^{32}P-labeled DNA. The chemiluminescence and hybridization signals were quantified by densitometry, and the antibody signal was adjusted to reflect the actual amount of DNA on the blot. The signal from 3 ng of 5 J/m^2 plasmid control was used to determine the number of CPDs in the genomic samples. Green and purple samples were compared by the permutation test; for 0 s, P = 0.0571; for 200 s, P = 0.885; for 500 s, P = 0.371; for 1000 s, P = 0.0285; and for 2000 s, P = 0.0285. One asterisk denotes a P value of ≤0.1; two asterisks denote a P value of ≤0.05.

TEV) was placed in adjacent slots, the signal from the UV-B-irradiated and UV-C-irradiated plasmid was not significantly different (data not shown). This means that at our sensitivity levels we do not see any difference in efficiency of antibody binding. Therefore, we used the UV-C-irradiated plasmid as the control in all our experiments with UV-B and UV-C.

We compared damage caused by UV-C in green and purple sheath tissue (Fig. 4A). The number of CPDs in green tissue is significantly greater (3-fold at 600 J/m^2 and 5-fold at 1200 J/m^2) than the number in purple tissue irradiated with the same UV-C dose. Although the DNA in green tissue was significantly more damaged than DNA in purple tissue, DNA inside plant cells is very well shielded from UV-C compared to naked DNA. For example, there were about 2×10^4 CPDs ng^{-1} J^{-1} m^{-2} in the green tissue sheath samples; in contrast, there were about 2×10^7 CPDs ng^{-1} J^{-1} m^{-2} in irradiated, purified DNA. This 1000-fold difference results from the poor penetration of UV-C into the multiple cell layers of the sheath tissue and to the absorption of UV-C by epidermal waxes or other surface components.

Do flavonoids protect during irradiation with the more physiologically relevant UV-B wavelengths? To test this, we measured CPD levels after irradiation with increasing amounts of UV-B (Fig. 4B). There is significantly more damage in the green samples than in the purple samples in the 1000-s and 2000-s UV-B doses. For the 2000-s green sample UV-B created 1.6×10^6 CPD/ng, and in the 2000-s purple sample it created only 8.0×10^5 CPD/ng. Thus, in sheath from greenhouse-grown purple and green plants exposed to additional UV-B radiation, we find that flavonoids offer some protection against the induction of CPDs. We next measured the steady-state levels of CPD in green and purple sheath and leaf samples harvested from field-grown plants early in the day (7:00 AM) and after full sun exposure (4:00 PM). We found no consistent differences in CPD levels between green and purple plants or between morning and afternoon samples (data not shown). However, there is more variation in the number of CPDs between individual sheath samples in field-grown plants than in sheath samples irradiated in the laboratory (data not shown). This variation is probably the result of differences in the amount of solar UV received by sheath tissue in the field.

In addition to CPDs, there is a second common UV-induced DNA photoproduct, the pyrimidine(6,4)pyrimidone. We used a monoclonal antibody specific to this photoproduct to de-

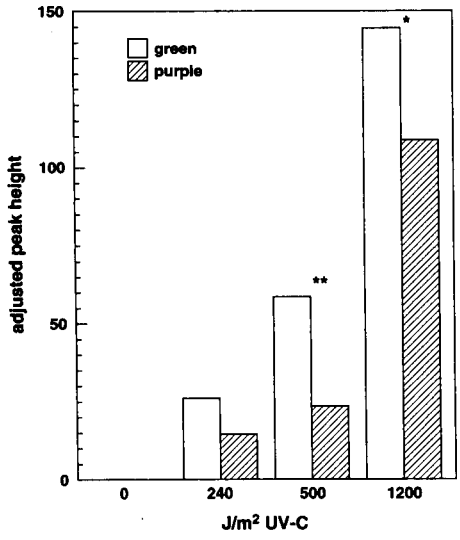

Figure 5. Pyrimidine(6,4)pyrimidone DNA damage in green (without anthocyanins) and purple (with anthocyanins) sheath tissue irradiated with UV radiation. Freshly excised sheath tissue was irradiated for 0, 5, or 10 s (0, 600, or 1200 J/m^2) with UV-C. The tissue was immediately frozen in liquid nitrogen, and DNA prepared as described in "Materials and Methods." Five hundred nanograms of genomic DNA were reacted with the TDM-2 antibody and the antibody signal was detected with chemiluminescence as described in "Materials and Methods." The amount of DNA in the antibody reactions was normalized by hybridization with ^{32}P-labeled DNA. The chemiluminescence signal and the hybridization signal were quantified by densitometry, and the antibody signal was adjusted to reflect the actual amount of DNA on the blot. One asterisk denotes a P value of ≤0.1; two asterisks denote a P value of ≤0.05. In the exposures that were used for densitometry the signal from 0 J/m^2 UV-C was undetectable.

termine the amount of pyrimidine(6,4)pyrimidone damage in irradiated sheath samples (Fig. 5). After irradiation with UV-C there was significantly more damage in the green samples than in the purple samples. We used UV-C rather than UV-B for the determination of the level of the 6,4 photoproduct because of a limitation in antibody specificity. The 64M-2 antibody recognizes only the 6,4 photoproduct, the major product generated by UV-C. It does not recognize the Dewar isomer of the 6,4 photoproduct; the Dewar isomer is produced by irradiation of the 6,4 photoproduct at 313 nm (UV-B), as would occur in our solar simulator (Cadet et al., 1992). Because we do not have a standard DNA sample with a known number of 6,4 photoproducts, we were unable to convert signal intensity to a precise number of 6,4 photoproducts.

DISCUSSION

After short irradiations with UV-C or UV-B sources there was less CPD DNA damage in maize plants that contain flavonoids than in plants that lack these compounds. This protective effect was seen using two different assays for CPD, the TEV assay and the antibody assay (Table I; Fig. 4, A and B).

After short irradiations with UV-C or UV-B there was no significant increase in the amount of CPD in purple tissues (Table I; Fig. 4, A and B). This suggests that the protective effect of flavonoids, as measured by CPD formation, has sufficient capacity to shield maize DNA entirely from CPD damage after short irradiations. However, there is a significant increase in the amount of 6,4 damage in purple tissue after 1200 J/m^2 of UV-C irradiation (Fig. 5). This suggests that the threshold for protection against the induction of the 6,4 photoproduct has been exceeded, although there is still significantly more 6,4 photoproduct in green tissue than in purple tissue.

We have shown that flavonoids can protect maize DNA from damage in vivo if the damage is generated by short UV exposure to excised tissue in the laboratory. We next wished to measure DNA damage levels in plants exposed to natural solar UV. Our antibody assay is sufficiently sensitive to measure the DNA damage levels we would expect from the UV levels found in sunlight. The steady-state level of UV-induced DNA damage in plants in the field is the sum of the amount of DNA damage induced by solar radiation and the amount of damage removed by repair of the DNA. We found no significant difference in steady-state CPD levels in field-grown green plants compared to purple plants. Because we know that induction of CPD is higher in green plants than in purple plants under laboratory conditions, our finding of no difference in CPD levels in the field highlights the complexity of analyzing the relative contributions of shielding and DNA repair. Possible explanations for our results include (a) increased CPD induction specifically in purple plants in the field or (b) increased repair of CPD in the green plants relative to the purple plants. We have no reason to think that the induction of CPD by UV radiation in the laboratory and induction of CPD by UV radiation in the field are different. There is, however, a rationale implicating differential repair of CPD.

In *A. thaliana*, accumulation of the key DNA repair enzyme, photolyase, which specifically monomerizes CPD, is induced by UV-B (Pang and Hays, 1991). If accumulation of photolyase in maize is also increased by UV-B, green tissues that receive more UV-B in the field may have higher levels of photolyase than purple tissues.

Another possibility is that photolyase levels are the same in the green and purple plants in the field but the function of photolyase is inhibited in purple plants. The net result would be more repair in green plants. One way in which photolyase function could be reduced in purple plants is if anthocyanins also absorb the light required for photolyase function. Photolyase enzymes require 375- to 400-nm light to provide the energy to split CPD (Sancar and Sancar, 1988). The mixture of anthocyanins present in purple maize tissues absorbs in this 375- to 400-nm spectral range in addition to the absorption in the UV-B range. Thus, it is possible that anthocyanins could filter out some of the light required for photolyase action. This seems unlikely, however, because there is much more energy in the 375- to 400-nm region of the sunlight spectrum than in the UV region of the spectrum (McLennan, 1987). Measurement of photolyase enzyme activity in green and purple plants from the field would distinguish between these possible explanations of the difference

between laboratory and field measurements of CPD in green and purple maize plants.

We have also measured levels of a second type of UV-induced DNA damage. We used a different monoclonal antibody to measure the levels of pyrimidine(6,4)pyrimidones in green and purple tissues. This antibody recognizes only 6,4 photoproducts, not the Dewar isomer of the 6,4 photoproducts. The Dewar isomer is produced by irradiation of the 6,4 photoproduct with 313-nm radiation (this is a wavelength in the UV-B region). Thus, we were unable to use UV-B to induce damage that is measurable with this antibody. Instead, we used UV-C. As we found when measuring CPD levels, after short irradiations there is more 6,4 photoproduct detectable in the green tissue than in the purple tissue (Fig. 5). This finding confirms that after short irradiations anthocyanins can protect maize DNA from UV-induced damage.

Flavonoids, including anthocyanins, perform diverse roles in angiosperms. During reproduction, anthocyanin pigments contribute to pollinator recruitment to flowers and to attraction of seed dispersal agents (Weiss, 1991). Anthocyanin is induced during various environmental stresses, including high light (McClure, 1975; Ryder et al., 1987; Feinbaum and Ausubel, 1988). Because the mixture of flavonoids found in plant tissues has a broad absorption spectrum in the UV-B, it has been hypothesized that these pigments shield plant DNA from UV-induced damage (Harborne, 1988; Stafford, 1990). We provide experimental support for this hypothesis by demonstrating that near isogenic green and purple tissues of maize accumulate different amounts of CPD after experimental UV-B irradiation. To quantify DNA damage after brief UV-B exposure, we developed highly sensitive antibody tests for two forms of DNA damage in plant samples. These new assays provide an accurate way to measure the kinetics of DNA damage and repair and will allow us to distinguish between possible explanations for the difference between induced CPD levels and steady-state CPD levels in greenhouse and field-grown green and purple maize plants.

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