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# Ultraviolet Light Inhibition of Phytochrome-Induced Flavonoid Biosynthesis and DNA Photolyase Formation in Mustard Cotyledons (*Sinapis alba* L.)[1]

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**In cotyledons of etiolated mustard (*Sinapis alba* L.) seedlings, phytochrome–far-red-absorbing form-induced flavonoid biosynthesis was found to be inhibited by short-term ultraviolet (UV) irradiations. UV inhibition was shown for the synthesis of quercetin, anthocyanin, and also for the accumulation of the mRNA for chalcone synthase, the key enzyme of this pathway. The UV effect was more pronounced on flavonoid biosynthesis, a process that selectively occurs in the epidermal layers, than on the synthesis of mRNA for chlorophyll *a/b*-binding protein localized in the mesophyll tissue. These UV inhibitory effects were accompanied by cyclobutane pyrimidine dimer (CPD) formation showing a linear fluence-response relationship. CPD formation and UV inhibition of flavonoid biosynthesis was found to be partially reversible by blue/UV-A light via DNA photolyase (PRE), allowing photoreactivation of the DNA by splitting of CPDs, which are the cause of the UV effect. Like flavonoid formation PRE was also induced by the far-red-absorbing form of phytochrome and induction was inhibited by UV. A potential risk of inhibition, in response to solar UV-B irradiation, was shown for anthocyanin formation. This inhibition, however, occurred only if photoreactivation was experimentally reduced. The PRE activity present in the etiolated seedlings (further increasing about 5-fold during light acclimatization) appears to be sufficient to prevent the persistence of CPDs even under conditions of high solar irradiation.**

Flavonoids such as flavones, flavonols, and also anthocyanins have been found to be induced by visible light or UV in a great variety of plant species (Beggs and Wellmann, 1994). Flavonoids are accumulated preferentially in the epidermal layers and show strong absorption in the UV. Therefore, these pigments have an important protective function against damaging effects from shorter wavelength solar radiation (Caldwell et al., 1983; Li et al., 1993; Beggs and Wellmann, 1994). The biosynthetic pathway of all classes of flavonoids (for review, see Heller and Forkmann, 1993) is initiated by the enzyme CHS (EC 2.3.1.74). CHS and other enzymes involved in flavonoid biosynthesis were studied in detail and serve as model systems for a better understanding of molecular aspects of gene regulation by light (Hahlbrock and Scheel, 1989; Batschauer et al., 1991).

In the environment, DNA is a sensitive target for UV damage mainly from the UV-B spectral region ($\lambda < 320$ nm) of solar radiation. In addition to avoidance of UV damage by means of shielding pigments, DNA repair also plays an essential role in UV protection. In higher plants two ways of DNA repair are known so far: excision repair, which includes endonucleolytic steps, base removal, and DNA re-synthesis (McLennan, 1987; Doetsch et al., 1989; Britt et al., 1993) and photorepair by PRE (EC 4.1.99.3). PRE monomerizes CPD in a light-dependent reaction (for review, see Sancar, 1994). PRE activity was shown in several plant species (Saito and Werbin, 1969; McLennan, 1987) and a PRE cDNA was cloned from mustard (*Sinapis alba* L.) showing light induction in re-etiolated leaves (Batschauer, 1993). Langer and Wellmann (1990) found PRE activity to be phytochrome regulated in bean hypocotyls. In *Arabidopsis thaliana* UV-B-induced DNA photorepair can be observed (Pang and Hays, 1990). Light induction of PRE has also been detected in other plant systems such as maize coleoptiles, parsley seedling roots and cell cultures, snapdragon seedlings, broad bean leaves (G. Buchholz and E. Wellmann, unpublished data), and mustard cotyledons as shown in this paper. Therefore, PRE can be assumed to be regulated within the framework of photomorphogenesis, as is flavonoid biosynthesis, with light induction of both UV-protective mechanisms playing an essential role in UV acclimatization during early developmental stages.

Wellmann et al. (1984) investigated the damaging effect of UV-B radiation on phytochrome-induced anthocyanin formation in mustard cotyledons. The action spectrum for this effect is similar to that of CPD formation (Matsunaga et al., 1991). Furthermore, this effect can be photoreactivated, also indicating a CPD-mediated process. A similar UV-B effect on anthocyanin formation in broom *Sorghum* seedlings was discovered by Hashimoto et al. (1991). These results indicate that CPDs mediate the effect. Induction of flavonoid biosynthesis by UV-B itself ($\lambda_{max}$ = 290–300 nm) is a common phenomenon in both etiolated and light-grown plants (Beggs and Wellmann, 1994). The action spectrum for the induction of flavonoid biosynthesis is shifted

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Abbreviations: CAB, Chl *a/b*-binding protein; CHS, chalcone synthase; CPD, cyclobutane pyrimidine dimer; PRE, photoreactivating enzyme DNA photolyase; T[]T, thymine dimer.

to longer wavelengths as compared to the action spectrum for its inhibition ($\lambda_{max}$ < 280 nm) (Wellmann et al., 1984; Hashimoto et al., 1991). Both action spectra are overlapping in the long-wavelength UV-B range and, therefore, a loss of synthesis of UV-B protective pigments might be expected if PRE activity is limiting.

In our experiments we have succeeded in analyzing the harmful effect of UV on the two known major light-induced UV-protective functions in the mustard cotyledons, namely the formation of flavonoid pigments and of PRE. Evidence will be presented for CPD damage in the DNA as a cause of this UV effect. UV inhibition of mRNA formation will be compared for CHS, which is located in the epidermal cells, and also for CAB, located in the mesophyll cells. Finally, the potential risk of solar UV damage to epidermal photoresponses is discussed, along with the essential role of PRE.

## MATERIALS AND METHODS

### Plant Material and Light Treatments

Seeds of white mustard (*Sinapis alba* L., harvest 1982) were obtained from Asgrow Company (Freiburg-Ebnet, Germany) and selected and sown as previously described (Mohr, 1966). Seedlings were germinated and grown in darkness at 25°C for 48 h. Only the larger cotyledon was used in all experiments. For UV irradiation, the cotyledons were cut in halves longitudinally at the midrib, placed on wet filter paper (Schleicher & Schuell 2034 BMGL, Dassel, Germany), and covered with a 3-mm quartz plate or 3-mm transmission cutoff filters of the WG series (Schott, Mainz, Germany) of $\lambda$ > 295, > 305, and > 335 nm, respectively (50% transmission at the given wavelengths). For light treatments, the cotyledon halves were arranged showing the underside. All of these manipulations were executed under a dim, green safelight (Mohr and Appuhn, 1963). The cotyledon halves were then subjected to the appropriate light treatment. For two-sided irradiation the half-cotyledons were turned over and irradiations were carried out at 25°C. Certain experiments were carried out at a reduced temperature as stated in the text. Irradiations with red and far-red light for the induction of phytochrome responses were carried out after the UV treatments.

### Light Sources

The red and far-red sources used in these experiments were as described by Schäfer (1977); far-red: $\lambda_{max}$ = 740 nm, half-bandwidth = 123 nm, fluence rate = 3.5 W $m^{-2}$; red: $\lambda_{max}$ = 660 nm, half-bandwidth = 18 nm, fluence rate = 6.7 W $m^{-2}$. Short-wavelength UV radiation was obtained from a Philips TL 40-W/12 fluorescent tube ($\lambda_{max}$ = 310 nm, half-bandwidth = 40 nm, fluence rate = 7 W $m^{-2}$). The spectral energy distribution of the lamp was 1% (0.01%) of $\lambda_{max}$ at 275 nm (254 nm). This light source was used unfiltered (covered with quartz) or filtered through 3-mm Schott, WG-type transmission cutoff filters (WG 295, 305, 335). UV-A was obtained from Osram L36-W/73 tubes (3.3 W $m^{-2}$) filtered by a 3-mm WG 335 transmission cutoff filter. For simultaneous irradiation with UV-B and UV-A, one TL 40-W/12 tube (2.2 W $m^{-2}$) was added to the UV-A field, and the samples were covered with a WG 295 transmission cutoff filter. Long-wavelength far-red light (RG9, fluence rate = 10 W $m^{-2}$) (Beggs et al., 1981) was obtained with a modified Leitz projector (Prado 500) equipped with Schott heat-absorbing glass (KG1) and a Schott RG9 glass filter (3 mm). Radiation measurements were carried out as described previously (Wellmann et al., 1984). The short-wavelength UV source was measured with a double monochromator spectroradiometer Optronic model 742 (Orlando, FL).

### Preparation of DNA Hydrolysates and T[]T-Specific Radioimmunoassay

Twenty cotyledon halves were ground for 3 min in 1.2 mL of DNA extraction buffer (0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 2% SDS [w/v], pH 7.6). The extracts were heated to 60°C and then centrifuged (5 min, 18,000*g*, 4°C). To 1 mL of the clear supernatants, 5 $\mu$L of RNase A (1 mg/mL, DNase free; Sigma, Deisenhofen, Germany) were added. After incubation for 30 min at 37°C the samples were mixed with an equal volume of phenol:chloroform (1:1, v/v), equilibrated with Tris-HCl (pH 7.6), and centrifuged (10 min, 14,000*g*, 4°C). Eight hundred microliters of the aqueous phase were removed, washed with an equal volume of chloroform, and once more centrifuged (5 min at 8000*g*). Out of 600 $\mu$L of the resulting aqueous phase, the DNA was precipitated by adding 60 $\mu$L of 3 M sodium acetate (pH 3.8) and 1.3 mL of ethanol and kept at −70°C for 20 min. The precipitates were pelleted by centrifugation (10 min at 18,000*g*) and redissolved in 1.5 mL of 90% formic acid. The solutions were sealed in glass tubes, placed in explosion-proof metal containers, and heated to 175°C for 75 min. The tubes were opened after freezing in liquid nitrogen. The samples were evaporated to dryness and dissolved in 100 $\mu$L of 0.15 M potassium phosphate, pH 7.0. A 50-$\mu$L aliquot was mixed with 25 $\mu$L of diluted antiserum (0.4 $\mu$L T[]T-specific antiserum [Klocker et al., 1982, 1984], 3 $\mu$L of preimmune serum in 21.6 $\mu$L of 0.15 M potassium phosphate) and 25 $\mu$L [*methyl*-$^3$H]T[]T (625 Bq, 3.8 TBq/mmol) in 0.15 M potassium phosphate. After incubation for 2 h at 4°C the proteins were precipitated by addition of 300 $\mu$L of a solution of 80% saturated ammonium sulfate (Green and Hughes, 1955) buffered with 0.15 M potassium phosphate, pH 7.0, for 30 min at 4°C. The precipitate was pelleted (5 min, 18,000*g*) and the supernatant was discarded. The pellet was dissolved in 100 $\mu$L of protein solubilizer (Soluene 350; Canberra Packard, Frankfurt, Germany) and radioactivity was determined by liquid scintillation counting with a toluene-based scintillation fluid (Rotiszint 11; Roth, Karlsruhe, Germany). The amount of T[]T binding was calculated from a standard curve for purified T[]T.

### RNA Extraction

Plant material was frozen in liquid nitrogen 6 h after the onset of irradiation and stored at −70°C. Total RNA was extracted from 40 cotyledon halves for each mea-

surement as previously described (Ehmann et al., 1991). Concentration and purity of RNA were determined spectrophotometrically.

### Labeling of the cDNAs

The gene-specific probes used for radioactive labeling were a 139-bp fragment of *Sinapis* CHS (SCHS-1) (Ehmann et al., 1991) and a 2.1-kb *Eco*RI fragment of a genomic clone encoding mustard CAB (Gauly et al., 1992). Radioactive labeling with [$\alpha$-$^{32}$P]dCTP (50 $\mu$Ci) was performed according to the manufacturer's protocol of the Megaprime labeling kit (Amersham Buchler, Braunschweig, Germany). Separation of free nucleotides from the labeled DNA was achieved by means of a Sephadex G-50 medium column with a 1.5-mL bed volume.

### RNA Dot Blots

Dilution series with RNA showed a maximum binding capacity of 6 $\mu$g of RNA per dot with the nitrocellulose filter used (type BA 85, Schleicher & Schuell). Thus, 6 $\mu$g of RNA were dotted for each measurement, as described previously (Ehmann et al., 1991). Dot blot filters were prehybridized for 1 h at 42°C. Hybridization was carried out for 36 h at 42°C. Filters were washed twice for 5 min in 2× SSC/0.2% (w/v) SDS at 42°C. For quantification, dots were cut from the nitrocellulose and filter-bound radioactivity was determined by liquid scintillation counting.

### Photolyase Assay

Forty half-cotyledons were ground for 2.5 min on ice with 800 $\mu$L of PRE extraction buffer (100 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA disodium salt, 10% glycerol, and 1 mM DTE) and 50 mg of quartz sand. The extract was centrifuged in a cooled desktop centrifuge (18,000*g*, 4°C) and the supernatant was passed through a buffer-equilibrated Sephadex G-25 fine spin column (Pharmacia), with a 5-mL bed volume. To determine photolyase activity, 570 $\mu$L of the eluate were mixed with 30 $\mu$L of a standard substrate solution (Langer and Wellmann, 1990) to a final substrate (T[]T) concentration of $3 \times 10^{-7}$ M. These manipulations were carried out under a yellow safelight (Osram L40W/62). The enzyme assay was incubated under photoreactivating conditions (UV-A source), and control samples were kept in darkness. The incubation temperature was 27°C. PRE extraction buffer (300 $\mu$L) was added to 500 $\mu$L of the assay mixture and the enzyme reaction was stopped by shaking with 800 $\mu$L of Tris-HCl equilibrated phenol: chloroform (1:1, v/v, pH 7.6). After the sample was centrifuged (10 min at 10,000*g*) 600 $\mu$L of the aqueous phase were removed, washed with an equal volume of chloroform, and centrifuged once more (5 min at 8000*g*). Out of 400 $\mu$L of the resulting aqueous phase, the DNA was precipitated with 40 $\mu$L of 3 M sodium acetate (pH 3.8) and 1 mL of ethanol at −70°C for 20 min. The precipitate (10 min at 18,000*g*) was dissolved in 90% formic acid. DNA hydrolysis and radioimmunoassay were performed as described above.

### Quercetin and Anthocyanin Determination

Anthocyanin was extracted and measured according to the method of Lange et al. (1971), if not otherwise noted, 24 h after the onset of irradiation, with 1 mL of the extraction medium for 15 cotyledon halves. For quercetin extraction, 48 h after the onset of irradiation, 15 cotyledon halves were heated to 95°C in 250 $\mu$L of ethanol:2 N HCl (1:1, v/v) for 1 h. After the sample was centrifuged, 200 $\mu$L of the supernatant were used for descending paper chromatography in 15% acetic acid. The region containing quercetin was eluted with 1.8 mL of ethanol and quantified spectrophotometrically at 372 nm.

## RESULTS AND DISCUSSION

Seedlings of white mustard have been intensively investigated for photomorphogenetic responses mediated by phytochrome (Mohr, 1983). One of these responses includes the formation of UV-absorbing flavonoids (Wellmann, 1974; Beggs et al., 1987), which function as a protection mechanism against UV damage (Tevini et al., 1991; Li et al., 1993; Beggs and Wellmann, 1994). In mustard cotyledons, anthocyanin biosynthesis can be induced to high levels by short-term irradiations with red light (Lange et al., 1971). On the other hand, flavonol biosynthesis depends on long-term far-red irradiations (high irradiance response; Beggs et al., 1987). Both pathways are spatially separated, with anthocyanin being accumulated in the lower epidermis and flavonols in the upper epidermis (Wellmann, 1974).

An inhibitory UV effect on anthocyanin formation and its reversibility by UV-A (photoreactivation) was previously described (Wellmann et al., 1984). This relationship applied also to the formation of the major flavonol aglycon quercetin (Fig. 1). Three minutes of UV irradiation on the upper side of the cotyledons led to a drastic decrease in the

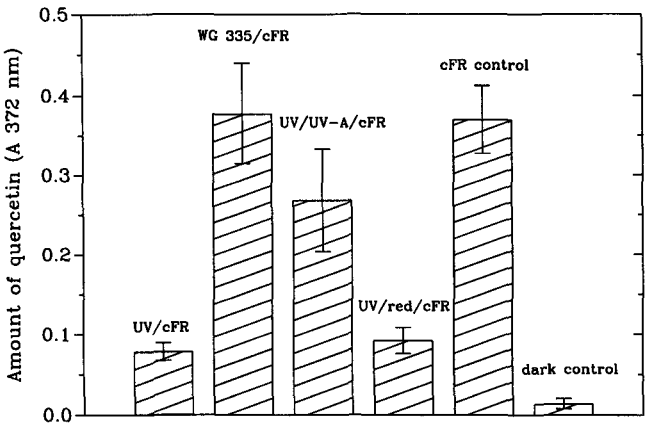


**Figure 1.** UV inhibition and photoreactivation of continuous far-red (cFR)-induced quercetin formation. Irradiations with light sources were: 3 min of UV; 3 min of UV covered with a 335-nm cutoff filter (WG 335); 3 min of UV plus 30 min of UV-A; 3 min of UV plus 30 min of red; controls for cFR and darkness. The cotyledon halves were irradiated on their upper sides. Afterward, the cotyledons were kept in continuous far-red for 48 h. Results are mean values ± SE from nine experiments.

effect of a subsequent, continuous irradiation with far-red light. This inhibitory effect proved to be reversible by UV-A irradiation immediately after the UV treatment, very probably as a consequence of repair by photolyase.

UV inhibition and photoreactivation of phytochrome-induced flavonoid biosynthesis could be detected at the transcript level for the key enzyme CHS, as shown in Figure 2A. This relationship was observed for the CHS transcript pool related to anthocyanin biosynthesis and for anthocyanin formation itself (Fig. 2B). The use of a red pulse for induction excluded experimentally flavonol biosynthesis and the related CHS transcript pool, depending on continuous far-red irradiation.

The fact that photoreactivation was not complete can be explained by UV damage of targets other than DNA, DNA damage other than CPDs, or photoreactivation being not effective enough to remove all CPDs.

To compare the UV effect on gene expression in different tissue layers of the cotyledon, transcript levels were determined for CHS and CAB, a nuclear-encoded plastid protein that is also formed in response to Pfr (Wenng et al., 1990). UV treatments were applied before the Pfr-activating irradiations. Long-term far-red irradiation (6 h) was used for optimal induction of both the anthocyanin- and the flavonol-related CHS mRNA pools (Fig. 3A) in the epidermal layers and CAB (Fig. 3B) in the mesophyll cells. UV-B fluences corresponding to 2 min of irradiation (1 min each on both sides of the cotyledons) resulted in a strong inhibitory effect on CHS mRNA formation, and after a treatment of 6 min (3 min each on both sides) at the same fluence rate, messenger levels were reduced to about 20% of the control excluding short-wavelength UV (Fig. 4A). Irradiations for 30 min were carried out with filtered UV only. In contrast,

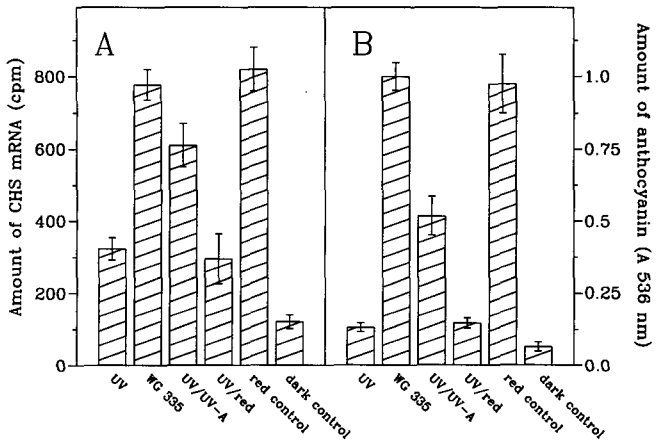


**Figure 2.** UV inhibition and photoreactivation of red-pulse-induced CHS transcript (A) and anthocyanin (B) formation. Irradiations with light sources were: 3 min of UV plus 5 min of red; 3 min of UV covered with a 335-nm cutoff filter (WG 335) plus 5 min of red; 3 min of UV plus 30 min of UV-A plus 5 min of red; 3 min of UV plus 30 min of red; controls for 5 min of red and darkness. Subsequent red pulses are given to adjust the same phytochrome photoequilibrium in all irradiation programs. Cotyledon halves were irradiated on their lower sides. Samples were kept in darkness for 6 h (A) and 24 h (B) after irradiation began. Results are mean values ± SE from four (A) and eight (B) experiments.

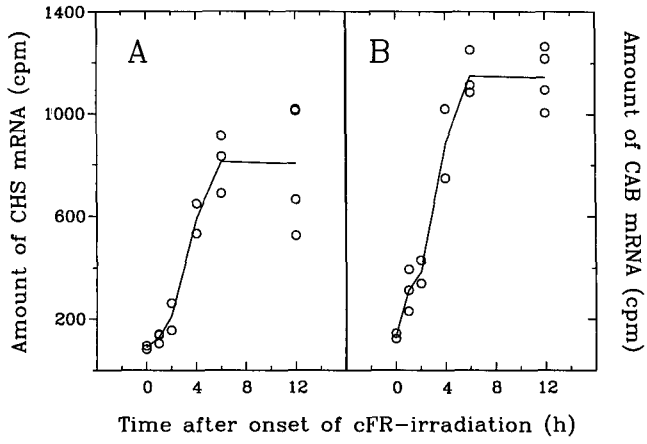


**Figure 3.** Time course of induction of CHS (A) and CAB (B) mRNA by continuous far-red (cFR). Cotyledon halves were irradiated on the upper side and frozen for analysis after the given times of irradiation. Results are mean values from two to four independent experiments.

the formation of CAB mRNA was not significantly affected even after high fluences (Fig. 4B). This difference in sensitivity toward UV is likely due to the tissue-specific expression of CHS in the epidermis and CAB in the mesophyll rather than to differences in copy numbers or lengths of the related genes. In mustard, CHS is present in a copy number of 4 (Batschauer et al., 1991), CAB is present in a copy number of 5 (A. Batschauer, personal communication), and the transcribed region of CHS (1923 bp) is about double the length compared to CAB (1054 bp), whereas the total length of the genes is nearly the same (CHS: 2873 bp, EMBL accession No. X16437; CAB: 2775 bp, EMBL accession No. X15894).

That the UV effects described in our experiments are not due exclusively to the short-wavelength part, which is no longer present in solar radiation can be concluded from irradiations under UV transmission cutoff filters at wavelengths of $\lambda > 295$ or $> 305$ nm (Fig. 4A). After increased irradiation times the inhibitory effect on CHS mRNA levels became visible even in response to wavelengths left after passing the 305-nm cutoff filter (about 1% transmittance left at 290 nm), whereas no response was observed on CAB mRNA levels (Fig. 4B). The relatively low risk of UV damage within the inner mesophyll tissue can be explained by screening from the outer cells containing proteins, phenolic compounds, or other UV-B-absorbing materials and also by the optical properties of the mesophyll itself, which contains storage protein in this early developmental stage.

Jordan et al. (1991, 1994) found a strong reduction of CAB transcript levels in pea leaves after supplementary UV-B irradiations. Unlike our results, however, a simultaneous induction of pigments and of CHS transcript was observed, leading to the conclusion that genes can be both inhibited and activated under UV-B stress. This holds true for legumes such as pea or bean, in which UV-B shows an elicitor function, inducing isoflavonoid formation under artificial radiation conditions with high fluences of short-wavelength UV-B (not present in solar light reaching the earth's surface). These effects can be reversed by photore-

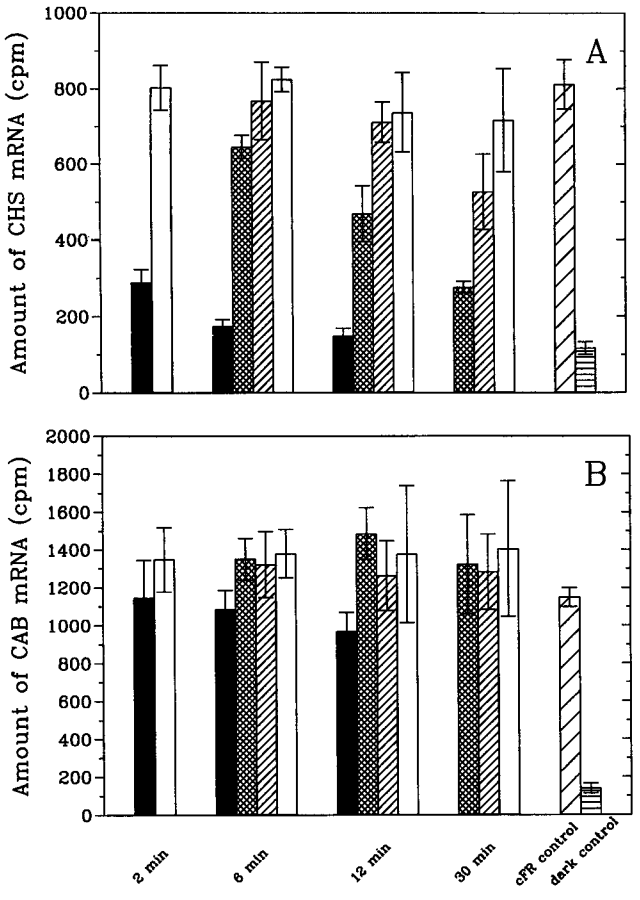


**Figure 4.** UV inhibition of Pfr-induced mRNA formation for CHS (A) and CAB (B) in dependence of the wavelength range and irradiation time. The cotyledon halves were covered either with quartz (■) or with transmission cutoff filters WG 295 (▩), WG 305 (▨), and WG 335 (□) and irradiated from both sides, each under the UV source for 1, 3, 6, or 15 min, followed by 6 h of continuous far-red (cFR). Controls for continuous far-red (▱) and darkness (▤). Results are mean values ± SE from four to eight experiments.

activation and are interpreted as a consequence of DNA damage (Beggs et al., 1985). Only flavonoid induction in epidermal cells by visible light via phytochrome and/or a blue-light photoreceptor or moderate solar UV via a postulated UV-B photoreceptor, respectively, can be considered a UV-protective reaction. This flavonoid induction would be totally inhibited under high UV-B irradiations capable of inducing isoflavonoid formation (Beggs and Wellmann, 1994).

UV inhibition of phytochrome-induced CHS mRNA synthesis or of flavonoid synthesis itself can be reversed by a subsequent UV-A irradiation allowing photoreactivation (Figs. 1 and 2). From this observation it can be assumed that CPDs are effective inhibitors of transcription (Sauerbier and Hercules, 1978). In our experiments to correlate the observed UV effects with direct measurements for T[]Ts it became apparent that only fluences strongly inhibiting flavonoid formation resulted in detectable amounts of T[]Ts (Fig. 5). UV damage could be detected with greater sensitivity by measuring the inhibition of flavonoid biosynthesis. Other assays for CPD detection (Mori et al., 1991; Quaite et al., 1994) may, however, be more sensitive when compared to our radioimmunoassay.

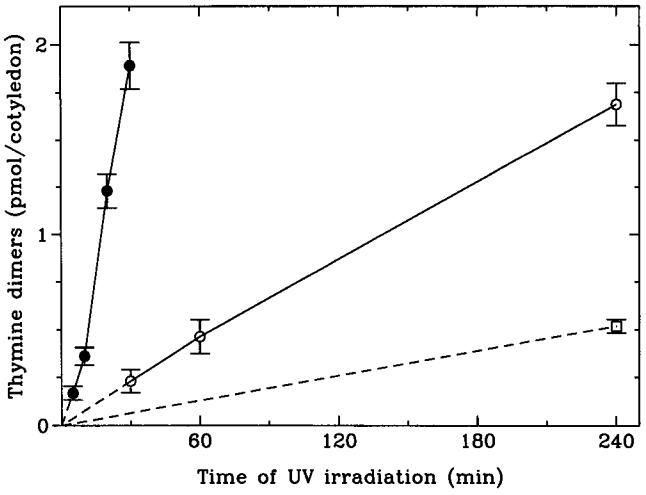


**Figure 5.** T[]T formation in response to different durations of UV irradiation. Cotyledons were irradiated from their lower sides under the UV source covered with quartz (●) or WG 295 at unreduced (○) and to one-third reduced (□) fluence rate. The amount of T[]T was determined just after irradiation. Results are mean values ± SE from four experiments.

Similar to biosynthesis of flavonoids, PRE activity was also increased in cotyledons of 48-h-old etiolated mustard seedlings as a response to continuous far-red light (Figs. 6 and 7A). The time course of enzyme induction suggests that this increased activity may result from de novo synthesis of the enzyme rather than from light activation of a precursor protein. At about 8 h after onset of irradiation, enzyme activity reached its maximum with a 5-fold increase over the dark control. The PRE activity changes in

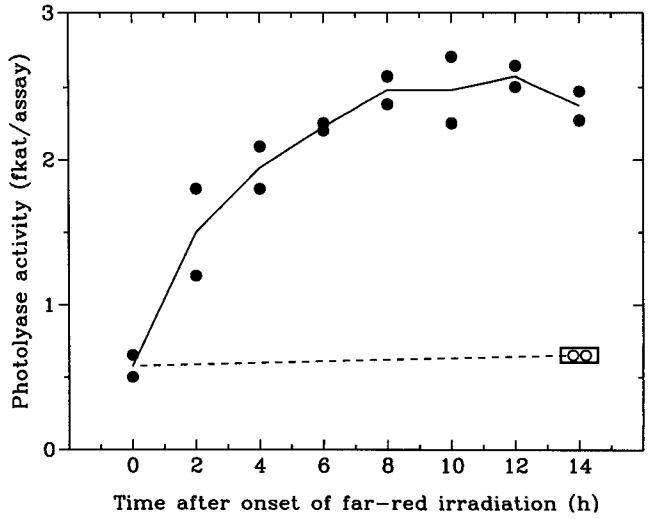


**Figure 6.** Induction of DNA photolyase in response to continuous far-red irradiation (●). Dark control (○). The cotyledons were irradiated from their lower sides. Results are from two independent experiments. kat, Katal (mol $s^{-1}$), unit for enzyme activity.

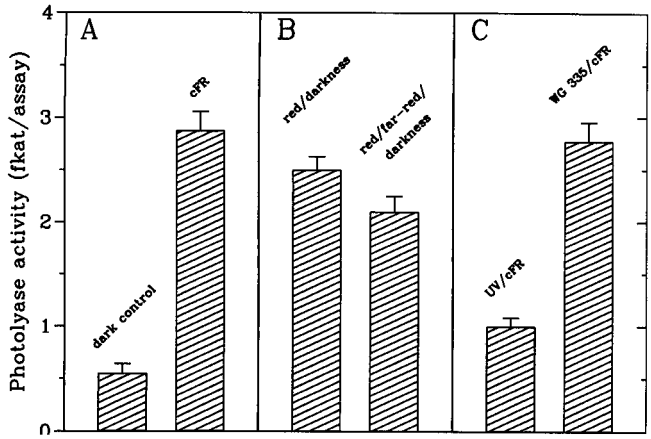


**Figure 7.** Effects of Pfr and UV on the induction of PRE. PRE activities were compared, each in corresponding cotyledon halves, for dark control and continuous far-red (cFR) (A); 5 min of red and 5 min of red plus 5 min of long-wavelength far-red (RG9) (B); 3 min of UV on each cotyledon side plus cFR and 3 min of UV covered with 335-nm transmission cutoff filter on each cotyledon side plus cFR (C). Continuous far-red was applied from the cotyledon lower side up to 12 h after the start of the irradiation program. PRE activity was determined 12 h after the start of the irradiation program. Results are mean values and SE from four experiments. kat, Katal (mol $s^{-1}$), unit for enzyme activity.

mustard described above are very probably the consequence of light-induced gene expression, recently measured at the RNA level in leaves of re-etiolated plants (Batschauer, 1993). Just as in bean hypocotyls (Langer and Wellmann, 1990) PRE was also strongly induced in mustard cotyledons by red-light pulses. This effect was photoreversed by about 20% (dark control subtracted) by long-wavelength far-red-light pulses, thus proving the involvement of phytochrome (Fig. 7B). Biosynthetic processes regulated via gene activation, typical for phytochrome action, could easily be inhibited by UV-B radiation as, for instance, by CPD formation. Evidence of this UV-B damage could also be detected in the inhibition of PRE induction by UV as shown in Figure 7C. UV irradiations of 3 min each of the upper and the lower side of the cotyledons preceding the far-red treatment resulted in a 60% reduction of the inductive effect, which was obtained with controls shielded with 335-nm transmission cutoff filters. The results shown in Figure 7C further suggest that light induction of PRE preferably occurs in the epidermal layer, because of UV inhibition being about as high as that of CHS mRNA and of pigment synthesis, respectively, but higher than that of CAB transcript. This conclusion would, however, require similar lengths and copy numbers of the compared genes, which is still unknown for PRE in mustard.

The essential role of PRE becomes obvious from the observation that a 75% restoration of UV-inhibited CHS transcript synthesis occurs within 30 min (Fig. 2), even starting from a low dark level of the enzyme. The capacity of this repair enzyme would be further increased in response to light (Figs. 6 and 7). Our results suggest that CPD formation represents the major UV damage in this plant system because of the high degree of UV-A photoreversibility of the inhibitory effects on flavonoid and CHS transcript formation. Dark repair processes concerning CPDs either have not occurred at all or are much less efficient than photoreactivation regarding restoration of CHS transcript formation during the experimental period of 6 h (Fig. 2A) or for flavonol formation during 48 h (Fig. 1).

Experiments to test the mustard cotyledons for PRE in vivo activity under long-term irradiations with UV-B-containing light showed that photoreactivation was strongly temperature dependent. This temperature effect can be explained by Pfr-induced PRE formation (Figs. 6 and 7), which is likely to be reduced at low temperatures rather than by a temperature dependence of the enzyme reaction. As shown in Figure 8, no significant temperature effect on T[]T accumulation, in response to UV-B alone, was observed, whereas simultaneous irradiations of UV-B with UV-A resulted in reduced amounts of T[]Ts, mainly at 25°C. No T[]Ts could be detected if a WG 335-nm transmission cutoff filter was used. Accordingly, the inhibitive UV-B effect on anthocyanin formation was found to be

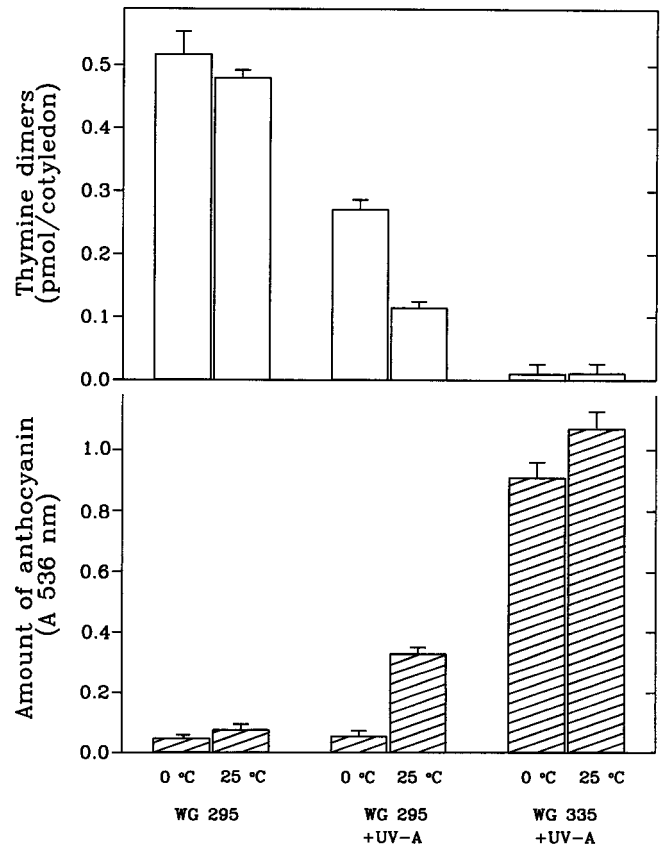


**Figure 8.** Temperature-dependent photoreactivation of T[]T and inhibition of anthocyanin formation. Cotyledon halves were incubated at 0 or 25°C, irradiated for 5 min with red light, kept in darkness for 2 h, and irradiated for 4 h with UV-B or UV-B simultaneously with UV-A. UV-B was filtered through WG 295. Controls were covered with WG 335. The amount of T[]T was determined just after the irradiation. For anthocyanin measurements the cotyledon halves were kept in darkness at 25°C for another 24 h. Results are mean values and SE of four (T[]T) and eight (anthocyanin) replicates.

partially reversed if PRE induction and photoreactivation were allowed at 25°C. Controls without UV-B treatment indicated that anthocyanin was only slightly reduced or delayed if the Pfr-activating light treatment was carried out under cooling.

To test to what extent the observed UV-B effects might also be relevant under natural radiation conditions, cotyledons were exposed to maximal solar UV for 4 h during the summer in Freiburg (48° N; 240 m altitude). Samples were cooled to inhibit Pfr induction of PRE and to keep photoreactivation at the lowest possible stage. Compared to controls with a UV-B transmission cutoff filter, up to 40% inhibition of anthocyanin formation in response to solar UV-B could be measured (see Table I). Cotyledons exposed without cooling showed no UV-B inhibition of anthocyanin formation. This plant system could be used as a basis for the evaluation of the potential risk of damage arising from solar UV-B increases as consequences of the decreasing stratospheric ozone layer (Kerr and McElroy, 1993). These effects can be extrapolated over a wide range of linear fluence-response relationships for UV-B inhibition, which could be demonstrated for anthocyanin formation (Wellmann et al., 1984) and also for CPD formation itself (Fig. 4).

Adverse effects from increasing solar UV-B should be considered, particularly during the acclimatization of plants to changing light environments, UV-B inhibiting the light-induced transcription of genes relevant for the synthesis of UV-protective functions, such as the formation of flavonoids and PRE. Our results support the view that these potentially UV-B-sensitive processes are well protected, with UV-B tolerance depending on the capacity of PRE. PRE activity seems to be high enough to compensate for CPD formation in the DNA arising even from unusually strong solar irradiations. No adverse effects in response to UV-B could be observed after solar irradiations over long periods, even in nonacclimatized, etiolated seedlings at normal temperature. In nature, germination would occur in the presence of some light allowing Pfr to induce flavonoid pigment formation and also higher levels of PRE before any exposure to full sunlight would be relevant. The situation might be more serious for plants showing UV acclimatization in response to UV itself via the postulated UV-B photoreceptor (Beggs and Wellmann, 1994) where wavelength ranges for pigment formation (Beggs and Wellmann, 1994) or PRE induction (Pang and Hays, 1991) and UV-B inhibition (Wellmann et al., 1984; Hashimoto et al., 1991) are overlapping. However, since there is no information about spectral fluence-response relationships of both adaptive and inhibitive responses in the same plant system, speculations concerning a calculable risk with regard to increases in solar UV-B after depletion of the ozone layer would be premature.

**Table I.** *Solar UV-B inhibition of anthocyanin formation*

Fifteen corresponding cotyledon halves were covered with 3 mm of quartz or 3 mm of WG 335-nm transmission cutoff filters (= controls), respectively, and exposed to sunlight. Cotyledon halves were kept on wet filter paper cooled to 5 or 25°C with a cryogenic system. Irradiations were carried out on clear days in Freiburg 48°N for 4 h (11:00 AM to 3:00 PM) during July and August. After light treatments the samples were kept in darkness for 24 h at 25°C. Results are mean values ± SE of eight parallels per experiment.

| Anthocyanin Formation | |
|---|---|
| 5°C | 25°C |
| *% of control* | |
| 57.5 ± 3.0 | 96.6 ± 2.5 |
| 74.5 ± 3.0 | 102.7 ± 2.1 |
| 77.6 ± 4.8 | 103.3 ± 2.3 |
| 71.4 ± 5.8 | 95.0 ± 2.6 |


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