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Effects of Light Fluence and Wavelength on Expression of the Gene Encoding Cucumber Hydroxypyruvate Reductase[1]

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We have investigated the regulation of cucumber (*Cucumis sativus*) hydroxypyruvate reductase mRNA abundance in response to white-, red-, and far-red-light treatments. Following irradiation of dark-adapted cucumber seedlings with 15 min to 4 h of either white or red light and return to darkness, the mRNA level for the gene encoding hydroxypyruvate reductase (*Hpr*) in cotyledons peaks in the darkness 16 to 20 h later. The response of the *Hpr* mRNA level to total fluence of white light depends more directly on irradiation time than on fluence rate. In addition to this time-dependent component, a phytochrome-dependent component is involved in *Hpr* regulation in dark-adapted green cotyledons as shown by red-light induction and partial far-red-light reversibility. Parallel measurements of mRNA levels for the ribulose bisphosphate carboxylase/oxygenase small subunit and for the chlorophyll *a*/*b*-binding protein show that *Hpr* is the most responsive to short (about 60 min) white- and red-light treatments and that each mRNA has a characteristic pattern of accumulation in dark-adapted cotyledons in response to light.

HPR (EC 1.1.1.29) is a peroxisomal enzyme that catalyzes the NADH-dependent conversion of hydroxypyruvate to glycerate in the process of photorespiration (Ogren, 1984). Photorespiration involves the light-dependent uptake of O_2 and release of CO_2 during the metabolism of phosphoglycolate, the two-carbon product of the oxygenase activity of Rubisco. During photorespiration, up to 75% of the carbon diverted from the Calvin cycle as phosphoglycolate is returned to the cycle as 3-phosphoglycerate in a process involving metabolite flow through the chloroplast, mitochondrion, and peroxisome (Husic et al., 1987).

Activities of HPR and other peroxisomal enzymes (glycolate oxidase and catalase) increase when etiolated wheat plants are transferred to white light (Feierabend and Beevers, 1972). This increase is a response to red, far-red, and blue wavelengths (Feierabend, 1975). HPR activity in etiolated cotyledons of *Pharbitis nil* also increases specifically in response to light of these wavelengths (Tchang et al., 1984). Our previous work showed that, in cotyledons of cucumber (*Cucumis sativus*), HPR activity, HPR protein, and translatable HPR mRNA are regulated both developmentally and by white light (Hondred et al., 1987).

We then isolated cDNA and genomic clones for a cucumber gene encoding HPR and showed that expression of the *Hpr* gene is limited to photosynthetic organs and is regulated developmentally and by white light (Greenler et al., 1989; Schwartz et al., 1991). A small, light-independent increase in *Hpr* mRNA is observed in etiolated cucumber cotyledons 4 to 5 d after germination (Greenler et al., 1989). Following, but not preceding, this developmental increase, cotyledons of dark-grown seedlings respond to 24 h of continuous illumination with a 5- to 10-fold increase in the *Hpr* mRNA level. When light-grown cucumber plants are transferred to darkness, *Hpr* mRNA abundance in leaves decreases more than 90% after 4 d of dark adaptation (Greenler and Becker, 1990). After the plants are returned to continuous white light, the *Hpr* mRNA level increases within 1 h and reaches the pre-dark level within 24 h.

Here we report effects of light fluence and wavelength on steady-state *Hpr* mRNA levels in green, dark-adapted cucumber seedlings and describe the kinetics of transcript appearance following light treatments. Levels of mRNA for *RbcS* and for *Cab* were also determined for comparison. Stimulation of expression of these genes by light is well documented and involves perception of red, far-red, and blue light (Mancinelli and Rabino, 1978; Tobin and Silverthorne, 1985; Dean et al., 1989; Quail, 1991; Thompson and White, 1991).

Because most studies of *RbcS* and *Cab* to date have focused on short duration (1–10 min) light treatments of etiolated plants, the kinetics of mRNA appearance for these genes in green plants is less well characterized. We show differences in transcript accumulation kinetics for cucumber *Hpr*, *RbcS*, and *Cab* and demonstrate that *Hpr* is the most responsive to white- and red-light treatments of 15 min to 2 h. We also describe the effects of red and far-red light on *Hpr* gene regulation and provide evidence for phytochrome involvement in regulation of *Hpr* gene expression in both dark-adapted and etiolated cucumber cotyledons. In addition, we demonstrate the usefulness of red and far-red LEDs in photobiological studies of plant gene induction and photoreversibility.

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Abbreviations: HPR, hydroxypyruvate reductase; *Hpr*, *RbcS*, *Cab*, and *Ubi*, the genes encoding hydroxypyruvate reductase, ribulose bisphosphate carboxylase/oxygenase small subunit, chlorophyll *a*/*b*-binding protein, and ubiquitin; LED, light-emitting diode.

MATERIALS AND METHODS

Plant Material

Seeds of cucumber (*Cucumis sativus* cv SMR 18) were sown at a depth of 1 cm in vermiculite or Jiffy Mix (Jiffy Products of America, Inc., Chicago, IL) that was saturated overnight with Hoagland solution. Seedlings were grown at 23 to 27°C under continuous white light for 5 d (cotyledons) or 12 d (leaves) and then transferred to darkness for a 4-d dark-adaptation period. Dark-adapted seedlings were irradiated as described for each experiment and then returned to darkness for 0 to 24 h for sampling as indicated. Experiments were performed once unless otherwise stated. Cotyledons or leaves were harvested directly into liquid nitrogen and stored at −50°C.

Light Sources

White light was supplied by a 4:1 mixture of Sylvania VHO cool-white fluorescent and 40-W incandescent bulbs at 160 or 270 μE m^{-2} s^{-1} of PAR (400–700 nm). Red and far-red components of this white light were provided using CBS red 650 and far-red 750 filters, respectively (Carolina Biological Supply, Burlington, NC). The red-light filter eliminated wavelengths below 600 nm, providing light with a peak fluence rate at 650 nm (19 μE m^{-2} s^{-1} between 600 and 700 nm). The far-red-light filter excluded wavelengths below 700 nm, providing light with a peak fluence rate at 762 nm (17 μE m^{-2} s^{-1} between 700 and 800 nm). The red filter also transmitted the far-red light described above. Arrays of LEDs (Quantum Devices, Barneveld, WI) provided red light (λ_{max} = 675 nm, 275 or 640 μE m^{-2} s^{-1}) or far-red light (λ_{max} = 746 nm, 450 μE m^{-2} s^{-1}) with half-bandwidths of 30 nm and did not require filters or special cooling. Measurements of light conditions were made using a Li-Cor LI-1800 portable spectroradiometer (Li-Cor Inc., Lincoln, NE). The following phytochrome photoequilibrium values (P_{fr}/P_{tot}) were calculated for these light sources as described by Sager et al. (1988): white light, 0.79; filtered red light, 0.71; filtered far-red light, 0.09; red LED light, 0.84, far-red LED light, 0.10.

RNA Isolation and Blotting

Frozen tissue was ground to a powder in liquid nitrogen and total RNA was prepared using the 4 M guanidinium isothiocyanate/phenol method of Chomczynski and Sacchi (1987). For each sample, total RNA was denatured in 50% formamide, 10 mM $NaPO_4$ (pH 6.8), 2.2 M formaldehyde, and 5 mM EDTA and then separated electrophoretically (10 μg/lane) in 1.0% agarose gels made using 10 mM $NaPO_4$ (pH 6.8). RNAs were blotted onto Nytran (Schleicher and Schuell) or Magnagraph (MSI, Westboro, MA) nylon membranes overnight in 10× SSC (Sambrook et al., 1989) and then covalently cross-linked to the membrane in a UV Stratalinker 1800 (Stratagene, La Jolla, CA).

Molecular Probes

The cucumber *Hpr* probe was a 1.4-kb *Eco*RI fragment from the cloned cDNA H18 (Greenler et al., 1989). The cucumber *RbcS* and *Cab* probes were 0.8-kb *Pst*I fragments from pCu5/1539 and pCu5/989, respectively (Greenland et al., 1987), and were provided by Dr. C. J. Leaver. The *Arabidopsis* ubiquitin (*Ubi*) probe was from Dr. Richard Vierstra. In all cases, insert DNAs were purified using a Geneclean II kit (Bio 101, La Jolla, CA) and labeled to a specific activity of about 1 × 10^9 cpm μg^{-1} using a randomly primed DNA-labeling kit (Boehringer Mannheim) and [^{32}P]dATP.

RNA Blot Hybridization and Analysis

Hybridizations were performed overnight at 42°C in 50% formamide, 5× SSPE (pH 7.5), 5× Denhardt's solution (Sambrook et al., 1989), 0.1% SDS, 0.1 mg mL^{-1} of denatured carrier DNA, and about 2 × 10^6 cpm of radiolabeled probe mL^{-1}. Membranes were then washed for 30 to 60 min in two to four changes of 0.1× SSC or SSPE, 0.1% SDS at 50 to 60°C for the cucumber probes and 25 to 35°C for the heterologous ubiquitin probe. Hybridization signals were quantified by direct β-counting of membranes using a Betascope 603 (Betagen Corp., Waltham, MA), followed by background correction, which, for the lowest measurement in each experiment, typically represented 30, 5, and 35% of total counts for *Hpr*, *RbcS*, and *Cab*, respectively.

RESULTS

Changes in mRNA Levels during Dark Adaptation

To characterize mRNA levels in dark-adapted leaves and to determine the optimal time for dark adaptation, cucumber seedlings were grown in continuous white light for 12 d until the first primary leaf was fully expanded. Samples of one leaf from each of three plants were taken immediately before (day 0) and every 24 h after transfer of plants to darkness for 6 d. Total RNA was prepared from these samples and assayed for *Hpr*, *RbcS*, *Cab*, and *Ubi* mRNAs by RNA blot hybridization. Figure 1a shows mRNA levels in leaves for each gene expressed relative to that in leaves from control plants harvested immediately before transfer to darkness. The amount of *Hpr* mRNA decreased to about 15% of the pre-dark control level within 24 h and to less than 10% by 2 d. The level of *RbcS* mRNA showed a similar but slightly slower decline. The *Cab* mRNA level was still 83% of control after 24 h and then decreased rapidly to less than 10% by the 3rd d in darkness. Although the *Ubi* probe was intended as a light-insensitive control, the amount of *Ubi* mRNA increased to 150% of the pre-dark control level during the first 24 h of dark adaptation and then gradually increased to more than twice the control level by the 4th and 5th d (Fig. 1a, inset).

To extend studies of *Hpr* expression to dark-adapted cotyledons and to test for possible organ-specific differences, we performed a similar experiment using fully expanded cotyledons from cucumber plants grown for 5 d in continuous white light before transfer to darkness for 0 to 6 d. The same patterns were observed in cotyledons as in leaves, although with slower initial declines for the three light-regulated mRNAs (Fig. 1b). All of the mRNAs declined to less than 10% of the pre-dark level in 3 to 4 d. The *Cab* mRNA level was stable for the first 24 h and then declined to 11% of the

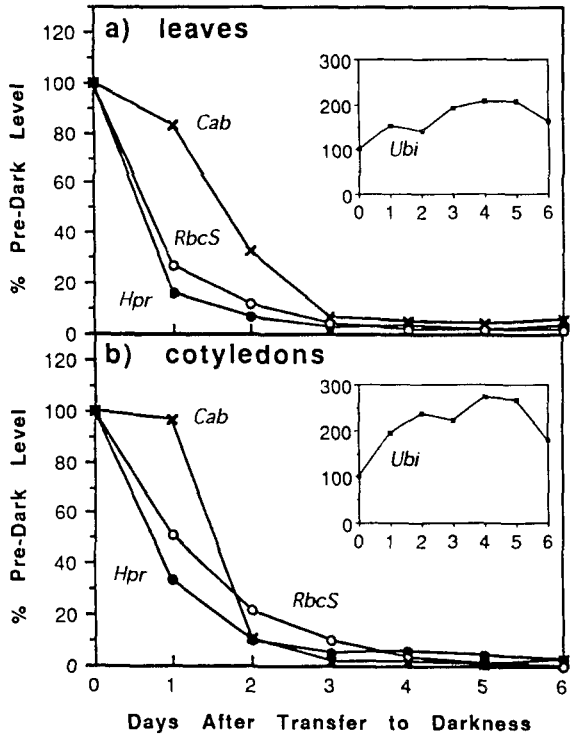

Figure 1. Changes in steady-state mRNA levels during dark adaptation of cucumber seedlings. a, Seedlings were grown in continuous white light (270 μE m^{-2} s^{-1}) for 12 d before transfer to darkness for 0 to 6 d. Expanded primary leaves from three seedlings were harvested every 24 h thereafter and were assayed for *Hpr*, *RbcS*, *Cab*, and *Ubi* (inset) mRNAs by RNA blot hybridization. The mRNA levels shown are expressed as percentages of the levels in control plants harvested before transfer to darkness. Axis labels for the inset graph are identical with those of the main graph. b, Seedlings were grown as above for 5 d in continuous white light and then transferred to darkness. Cotyledons were harvested and assayed for mRNAs as described for leaves.

pre-dark level by the 2nd d. As in leaves, the *Ubi* mRNA level in cotyledons increased substantially during dark adaptation to 280% of the pre-dark level by d 4 (Fig. 1b, inset). Although dark-adapted leaves appeared healthy throughout the experiment, dark-adapted cotyledons began to senesce by the 6th d. Therefore, 4 d was chosen as the optimal dark adaptation period for further studies with both leaves and cotyledons.

Kinetics of mRNA Accumulation in Response to Continuous White Light

Dark-adapted cucumber seedlings were transferred to continuous white light for sampling of cotyledons every 4 h from 0 to 40 h. The accumulation kinetics of *Hpr*, *RbcS*, and *Cab* mRNAs differed considerably upon return of the seedlings to white light (Fig. 2). The *Hpr* mRNA level increased rapidly to 8.5 times the dark-adapted control level within 4 h and showed a further increase by 20 h to a peak of 42 times control. The *RbcS* mRNA level increased less dramatically, reaching a peak of only 7.5 times control, which occurred after 28 h of continuous white light. The *Cab* mRNA response differed from either of the others, showing a rapid increase to 42 times control by 8 h, a sharp decrease at 16 h, and a second rapid increase to 48 times control by 28 h.

Kinetics of mRNA Accumulation in Response to Shorter Duration White-Light Treatments

To determine whether continuous illumination was necessary for this light-dependent increase in *Hpr* mRNA level, dark-adapted seedlings were irradiated with white light for 1 h and returned to darkness, and cotyledons were harvested every 4 h from 0 to 24 h. In each of three such experiments, a reproducible peak in *Hpr* mRNA level occurred in the darkness 16 to 20 h following white-light treatment (Fig. 3), representing an 8-fold increase over the level in dark-adapted controls. Although the peak was lower in magnitude than in continuous white light (Fig. 2), its timing was the same, demonstrating that qualitative aspects of the *Hpr* response following short duration white-light treatments proceed in the absence of continued illumination.

To assess the effects of irradiation time on mRNA accumulation, dark-adapted seedlings were irradiated with white light (fluence rate = 160 μE m^{-2} s^{-1}) for 15 min, 30 min, 1 h, or 2 h and returned to darkness. Cotyledons were harvested every 4 h thereafter and analyzed for *Hpr*, *RbcS*, and *Cab* mRNAs (Fig. 4). Of the three genes tested, *Hpr* showed the greatest increase in mRNA level relative to the dark-adapted control following these shorter treatments. For each irradiation time, the peak level of *Hpr* mRNA was seen after 16 to 20 h of subsequent darkness, with the peak levels approxi-

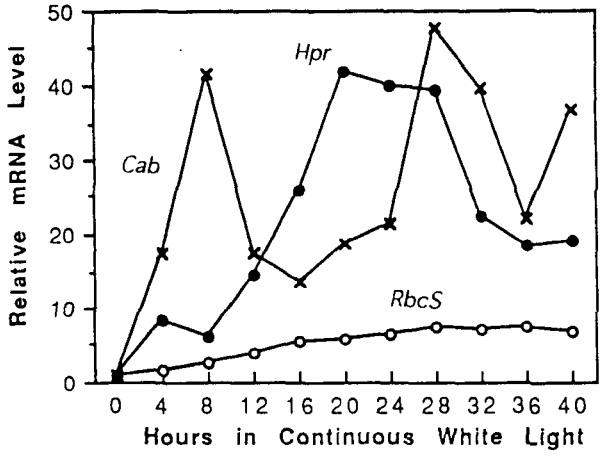

Figure 2. Kinetics of mRNA accumulation in dark-adapted cucumber cotyledons in response to continuous white light. Seedlings were grown in continuous white light (160 μE m^{-2} s^{-1}) for 5 d, transferred to darkness for 4 d, and then returned to continuous white light. Cotyledons were harvested immediately before transfer to continuous white light and every 4 h for 40 h thereafter and were assayed for *Hpr*, *RbcS*, and *Cab* mRNA levels. The mRNA levels shown are expressed relative to those in dark-adapted cotyledons harvested before irradiation. Peak levels for each mRNA are approximately the same upon reillumination as in continuous white-light-grown plants that were not dark adapted.

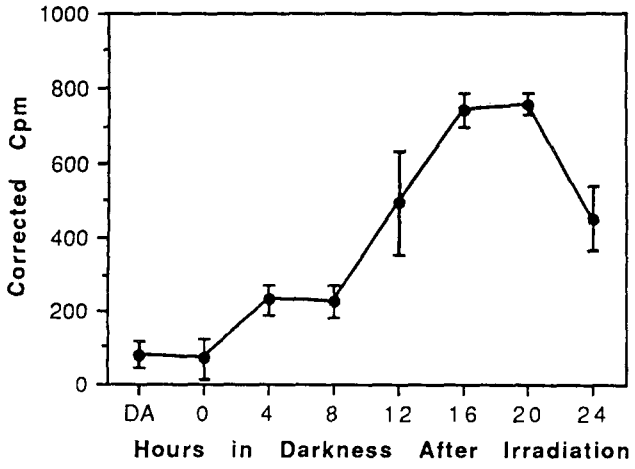

Figure 3. Kinetics of *Hpr* mRNA accumulation in dark-adapted cotyledons in response to 1 h of white light. In three independent experiments, dark-adapted seedlings were irradiated with white light (160 μE m^{-2} s^{-1}) for 1 h and then returned to darkness for 0 to 24 h. Cotyledons were harvested after 4 d of dark adaptation (DA), immediately following irradiation (0 h darkness), and every 4 h after return to darkness and were assayed for *Hpr* mRNA. To determine experimental reproducibility, the RNA blots from these experiments were hybridized together with the same radiolabeled *Hpr* probe. The mRNA levels shown are expressed as total cpm hybridized after background correction (about 50 cpm), and data points represent the means ± SD in each case.

mately proportional to irradiation times. As shown earlier for continuous white-light treatment, the *RbcS* mRNA level was less responsive to shorter treatments than that of *Hpr*, since 2 h of irradiation was necessary to cause a detectable increase over the dark control level. The level of *Cab* mRNA also responded less to these shorter duration white-light treatments than did that of *Hpr*. A peak in *Cab* mRNA level of 4 times control was seen after 2 h of irradiation and 4 h of darkness and a second peak was seen 12 to 16 h later.

Peak mRNA values from each curve in Figure 4 were plotted as a function of irradiation time in Figure 5a, along with data from an otherwise identical experiment in which the irradiation time was increased to 4 h. The peak *Hpr* mRNA level was proportional to irradiation time (and, therefore, also to total fluence because the fluence rate was constant). Both *RbcS* and *Cab* mRNA levels required about 2 h of illumination for a significant response. Thereafter, a doubling of the irradiation time from 2 to 4 h resulted in a doubling of the peak mRNA level for *RbcS*, and that of *Cab* increased about 10-fold.

The proportionality of peak *Hpr* mRNA level to irradiation time suggested that the response was a direct function of either irradiation time or total fluence (i.e. fluence rate × time). To test the hypothesis that the response was to total fluence, we varied total fluence by changing fluence rate rather than irradiation time. This hypothesis predicts that the relationship between peak *Hpr* mRNA level and total fluence will be the same as when irradiation time is varied. Dark-adapted seedlings were irradiated for 1 h with white light at 40, 80, 160, or 300 μE m^{-2} s^{-1} and then returned to darkness for sampling of cotyledons every 4 h thereafter. As fluence rate increased by a factor of 7.5 from 40 to 300 μE m^{-2} s^{-1}, the *Hpr* mRNA level increased less than 2-fold (Fig. 5b). These results do not support the above hypothesis and suggest that the peak *Hpr* mRNA level is not linear with respect to total fluence but responds more directly to irradiation time than to fluence rate. Moreover, a fluence rate of 300 μE m^{-2}

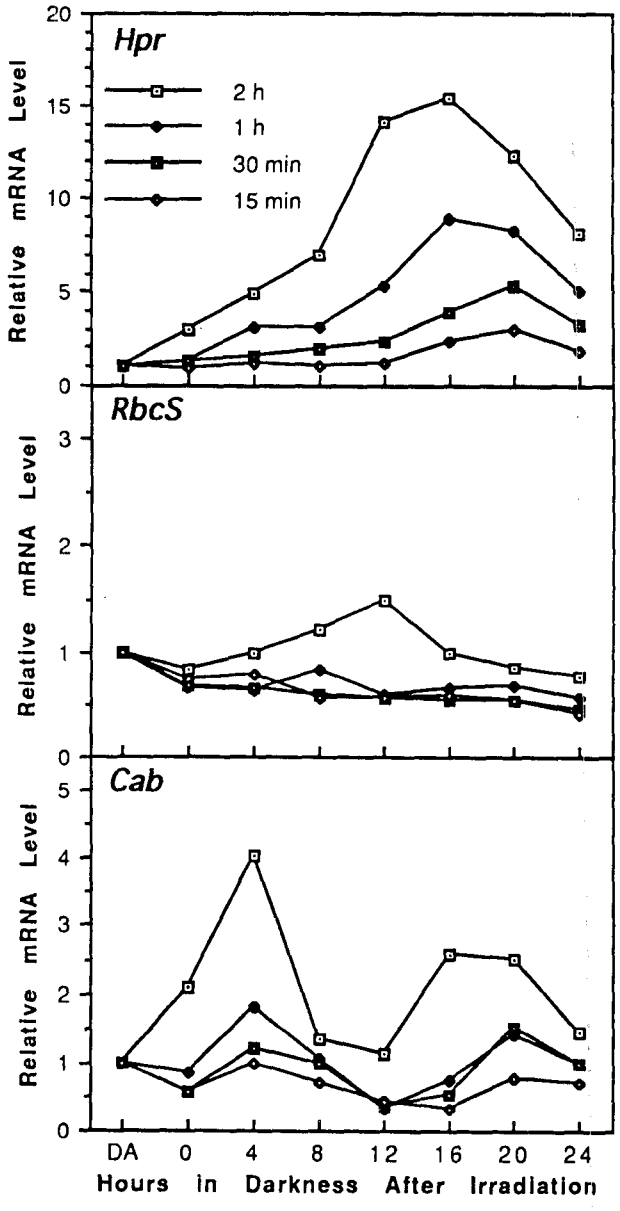

Figure 4. Kinetics of mRNA accumulation in dark-adapted cucumber cotyledons in response to white light. Dark-adapted seedlings were irradiated with white light (160 μE m^{-2} s^{-1}) for 15 min, 30 min, 1 h, or 2 h and then returned to darkness for 0 to 24 h. Cotyledons were harvested immediately following irradiation (0 h darkness) and every 4 h for 24 h thereafter and were assayed for *Hpr*, *RbcS*, and *Cab* mRNAs. The mRNA levels shown are expressed relative to those in dark-adapted cotyledons harvested before irradiation (DA). Note the different scales on the ordinates of the three panels.

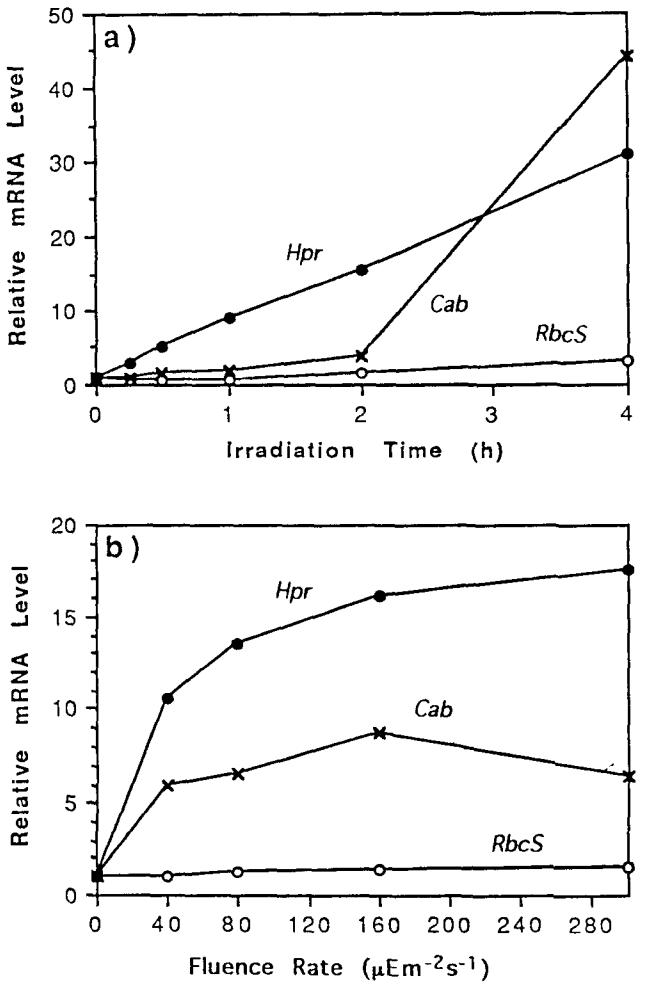

Figure 5. Response of peak mRNA levels to changes in total fluence. Dark-adapted cucumber seedlings were irradiated with white light and returned to darkness for sampling of cotyledons every 4 h for 24 h. Cotyledons were assayed for *Hpr*, *RbcS*, and *Cab* mRNAs, and peak levels were determined relative to the levels in dark-adapted control seedlings. a, Total fluence was varied by irradiation with white light (160 μE m^{-2} s^{-1}) for 15 min, 30 min, 1 h, 2 h, or 4 h. b, Total fluence was varied by irradiation for 1 h with white light at 40, 80, 160, or 300 μE m^{-2} s^{-1}.

s^{-1} appears to be near saturating for the *Hpr* response to a 1-h irradiation.

Effect of Light Quality on mRNA Accumulation

To determine the contribution of red light to *Hpr* mRNA accumulation, dark-adapted seedlings were irradiated for 4 h with either white light or filtered red light (see "Materials and Methods"), and cotyledons were sampled immediately. The level of *Hpr* mRNA increased approximately 6-fold following both white- and red-light treatments, indicating a significant red-light effect (Fig. 6). *RbcS* also responded to white light and red light but with only a doubling of mRNA level. The level of *Cab* mRNA was increased maximally by white light but only moderately by red light, indirectly suggesting a greater blue-light requirement for *Cab* versus *Hpr* in dark-adapted cucumber cotyledons.

Far-Red Reversibility in Green Seedlings

The effectiveness of red-light treatments in mimicking the effect of white light on *Hpr* mRNA accumulation suggested phytochrome involvement in regulation of *Hpr* mRNA levels in dark-adapted cucumber seedlings. To test this hypothesis, dark-adapted seedlings were irradiated for 4 h with either white light or filtered red light or were left untreated. Thereafter, half of each group was returned to darkness, and the other half received 60 min of filtered far-red light, followed by return to darkness. Cotyledons were harvested 16 h after transfer to darkness and were assayed for *Hpr*, *RbcS*, and *Cab* mRNAs.

For all three genes, subsequent far-red-light treatment led to significant inhibition of mRNA accumulation 16 h following white-light or red-light treatment (Fig. 7). Results from this experiment and from an analogous experiment using dark-adapted leaves (data not shown) are summarized in Table I. This table shows the percentage decrease in mRNA levels in both organs caused by subsequent far-red-light treatment and demonstrates a phytochrome component in the responses. All three genes show partial far-red inhibition of white-light- and red-light-dependent mRNA accumulation in green, dark-adapted cucumber seedlings, an effect not previously demonstrated for *Hpr*.

Irradiation using Red LEDs

Levels of *Hpr* mRNA were similar immediately after irradiation with 4 h of either white or filtered red light (Fig. 6), but the effect of white light was 6-fold greater than that of red light following transfer to darkness for 16 h (Fig. 7, top). This could indicate that the filtered red light lacks a compo-

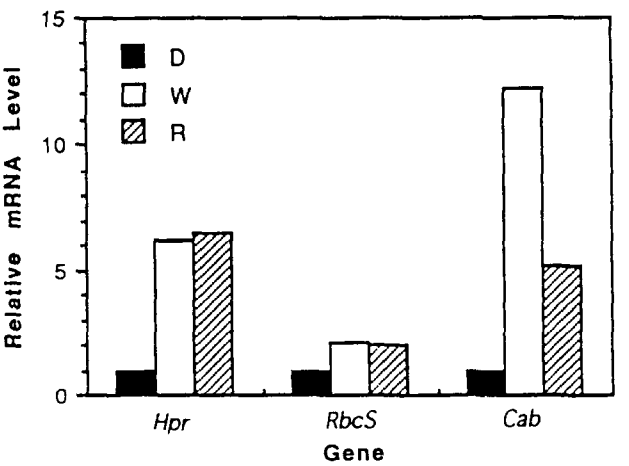

Figure 6. Effect of white and red light on mRNA accumulation in dark-adapted cucumber cotyledons. Dark-adapted seedlings were left untreated or were irradiated for 4 h with either white light (W; 270 μE m^{-2} s^{-1}) or filtered red light (R; 19 μE m^{-2} s^{-1}). Cotyledons were harvested immediately and assayed for *Hpr*, *RbcS*, and *Cab* mRNAs. The mRNA levels shown are expressed relative to those in untreated, dark-adapted cotyledons (D).

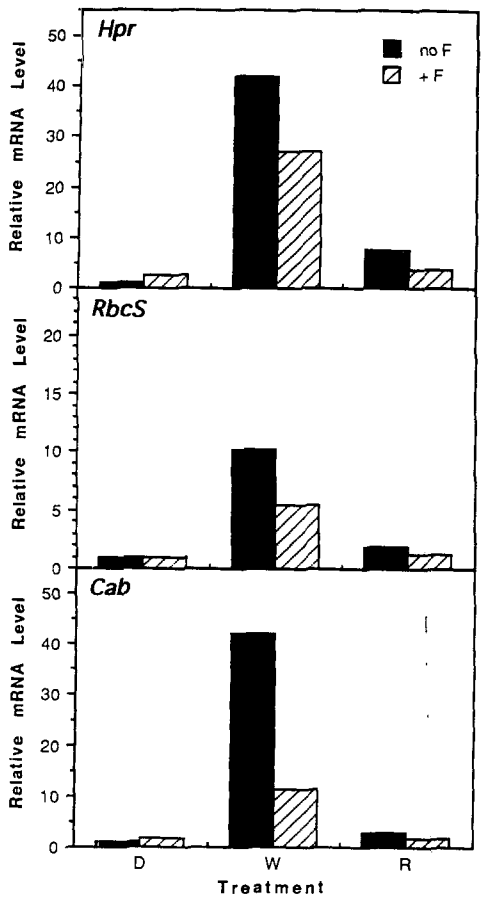

Figure 7. Effect of far-red light on mRNA accumulation in dark-adapted cucumber cotyledons following white- or red-light treatment. Dark-adapted seedlings were left untreated or were irradiated for 4 h with either white light (W; 270 μE m^{-2} s^{-1}) or filtered red light (R; 19 μE m^{-2} s^{-1}). Immediately thereafter, half of each group (no F) was returned to darkness and the other half (+F) was irradiated for 60 min with filtered far-red light (17 μE m^{-2} s^{-1}) before return to darkness. Cotyledons were harvested 16 h thereafter and assayed for *Hpr*, *RbcS*, and *Cab* mRNAs. The mRNA levels shown are expressed relative to those in dark-adapted, untreated plants harvested concurrently (D, no F). Note the different scales on the ordinates of the three panels.

nent present in white light that is responsible for the continued increase in darkness. Alternatively, the far-red-light component present in the filtered red light could be inhibitory.

To assess more adequately the role of red light in *Hpr* mRNA induction, we used red LEDs as an alternative to the red filter described previously. These LEDs emit intense, nearly monochromatic red light and generate little heat, thereby eliminating the need for filters or special cooling (Bula et al., 1991; Barta et al., 1992). Dark-adapted seedlings were irradiated for 1 or 2 h using red LEDs and then returned to darkness for 0 to 24 h for sampling of cotyledons and mRNA analysis. As seen earlier with white light, the *Hpr* mRNA level increased dramatically in response to either 1 or 2 h of irradiation with red LEDs, reaching peaks 16 h later that were approximately proportional to irradiation time (Fig. 8). The *Cab* mRNA level also increased markedly following the 2-h irradiation with red LEDs, showing peaks at 4 and 16 h after irradiation. The 1-h treatment was only about 25% as effective as the 2-h treatment. As seen in previous experiments using white light and filtered red light, the *RbcS* mRNA level was the least responsive of the three examined. These results show that red light initiates a process that proceeds in the absence of continued light and leads to a large increase in the *Hpr* mRNA level. Therefore, the lack of a similar increase seen using filtered red light was most likely due to the inhibitory far-red light also transmitted by the red filter.

To determine whether shorter irradiation times with red light would increase mRNA levels for these genes in dark-adapted cotyledons, we tested the effects of a 5- or 30-min treatment using red LEDs. To test for phytochrome involvement, some seedlings were given a 5- or 30-min red-LED treatment and a subsequent far-red-LED treatment of equal duration. All seedlings were then returned to darkness and assayed for *Hpr*, *RbcS*, and *Cab* mRNAs 16 h later. A 5-min irradiation using red LEDs had little effect on mRNA levels for any of the three genes in dark-adapted cotyledons (data not shown). For the three genes tested, only the *Hpr* mRNA level was increased by the 30-min red-light treatment relative to the dark-adapted control, and this increase was inhibited approximately 50% by the subsequent far-red-light treatment (data not shown).

Photoreversibility in Etiolated Cotyledons

To compare *Hpr* mRNA accumulation in dark-adapted versus etiolated cotyledons and to assess the applicability of LEDs in photoreversibility studies, we repeated the above analysis with etiolated plants. Cucumber seedlings were grown for 5 d in darkness, given 5- or 30-min LED irradiations as described above for dark-adapted seedlings or a white-light treatment of equal duration, and then transferred to darkness for 16 h before sampling. In contrast to the minimal effects observed in green, dark-adapted cotyledons, both red-LED treatments increased the *Hpr* mRNA level 2 to 3 times above nonirradiated controls to a level comparable to that measured following white-light treatments of the same duration (Fig. 9). A 30-min far-red-light treatment following 30 min of red light somewhat inhibited the mRNA increase produced by the red-light treatment alone. After 5 min of red

Table I. *Percentage inhibition of mRNA accumulation by far-red light following irradiation with white or red light*

These values are derived from the experiment shown in Figure 7 and from an analogous experiment using dark-adapted leaves (data not shown). Values represent percentages of decrease in mRNA levels of far-red-light-treated cotyledons (cots) or leaves (lvs) relative to the mRNA level (100%) of corresponding organs not receiving far-red light.

Light	*Hpr*		*RbcS*		*Cab*	
	Cots	Lvs	Cots	Lvs	Cots	Lvs
White	36	58	48	53	73	81
Red	52	71	33	50	43	78

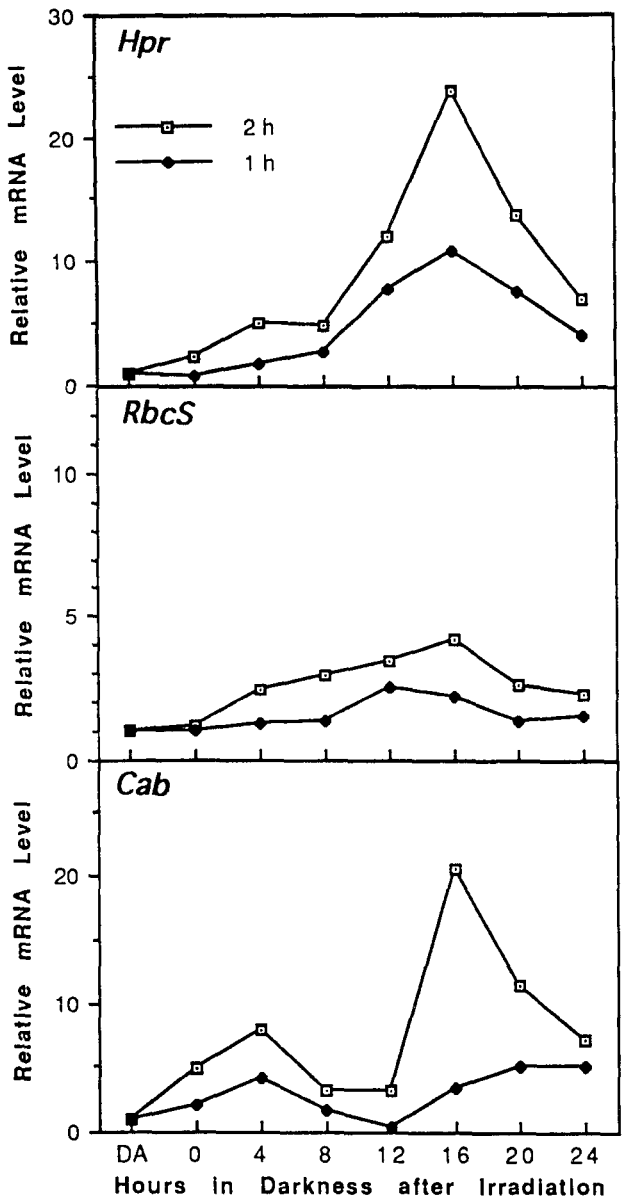

Figure 8. Kinetics of mRNA accumulation in dark-adapted cucumber cotyledons following irradiation using red LEDs. Dark-adapted seedlings were irradiated (275 μE m^{-2} s^{-1}) for 1 or 2 h and then returned to darkness for 0 to 24 h. Cotyledons were harvested every 4 h and were assayed for *Hpr*, *RbcS*, and *Cab* mRNAs. The mRNA levels shown are expressed relative to those in dark-adapted cotyledons (DA). Note the different scales on the ordinates of the three panels.

light, however, 5 min of far-red light reduced the *Hpr* mRNA level to that produced by far-red light alone. None of the treatments caused any greening of the cotyledons, thereby eliminating any photosynthetic component of *Hpr* expression. These results demonstrate phytochrome involvement in *Hpr* expression in etiolated cotyledons and indicate an escape time of between 5 and 30 min for photoreversibility of *Hpr* gene expression in dark-grown cotyledons. To our knowledge, this represents the first use of red and far-red LEDs in studies of plant gene induction and photoreversibility.

As in dark-adapted cotyledons, an increase of the *RbcS* mRNA level in etiolated cotyledons by either white light or red LED light was less than that of either *Hpr* or *Cab*. Because of the low level of response of *RbcS* mRNA levels, photoreversibility could not be demonstrated. Whereas the response of *Cab* to 5 min of white light or red light was similar in magnitude to that of *Hpr*, 30-min treatments led to considerably higher levels. Some response to 5 or 30 min of far-red light alone was seen, but far-red light following red light reduced the *Cab* mRNA level to that seen after far-red light alone in both cases. This observation suggests a longer escape time for photoreversibility of *Cab* versus *Hpr* gene expression in etiolated cucumber cotyledons.

DISCUSSION

We previously showed that the *Hpr* mRNA level is high in primary leaves of cucumber seedlings grown in continuous

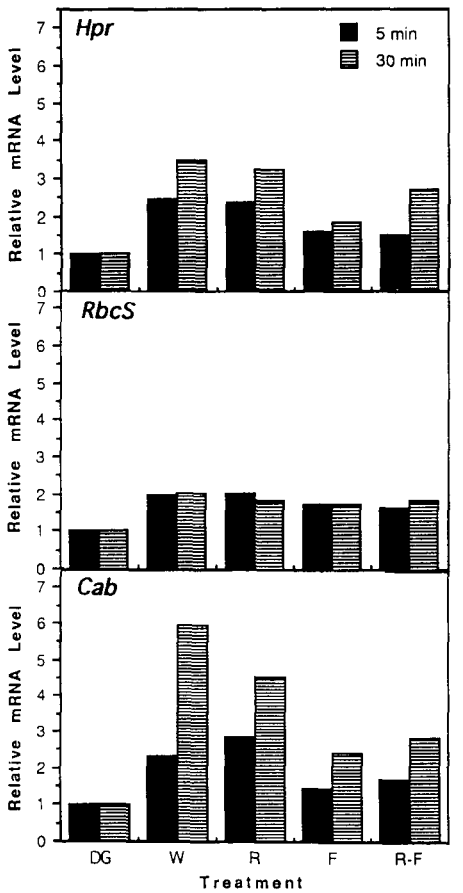

Figure 9. Demonstration of photoreversibility in etiolated cucumber cotyledons using red and far-red LEDs. Seedlings were grown in darkness for 5 d and were then left untreated or irradiated for 5 or 30 min with white light (W; 160 μE m^{-2} s^{-1}), red light (R; 640 μE m^{-2} s^{-1}), far-red light (F; 450 μE m^{-2} s^{-1}), or red followed by far-red light (R-F) (i.e. 5 min R + 5 min F or 30 min R + 30 min F). Plants were returned to darkness for 16 h, and then cotyledons were harvested and assayed for *Hpr*, *RbcS*, and *Cab* mRNA levels. The mRNA levels shown are expressed relative to those in dark-grown, nonirradiated cotyledons (DG).

white light, decreases more than 90% during 4 d of dark adaptation, and gradually increases to the pre-dark level within 24 h of transfer to continuous white light (Greenler and Becker, 1990). Here we have extended the analysis to dark-adapted cotyledons and have shown that 2 h of white light (Fig. 4) or red light (Fig. 8) followed by 16 h of darkness are sufficient to increase *Hpr* mRNA levels 15- to 25-fold above nonirradiated controls. This is the approximate level seen after 16 h of continuous white light (Fig. 2). Continuous irradiation is not required during most of this increase, suggesting that an inductive response is triggered by a short duration, initial light treatment. Each doubling of irradiation time at constant fluence rate led to a doubling of peak *Hpr* mRNA level (Figs. 4 and 5a). However, a 7.5-fold increase in fluence rate at constant irradiation time led to less than a 2-fold increase (Fig. 5b). These results suggest greater involvement of a time-dependent rather than fluence rate-dependent component in regulating the magnitude of the *Hpr* mRNA level increase. This time-dependent component may be due to the stimulatory effect of a photosynthetic product that is produced at a constant rate during irradiation. This possibility is supported by our previous demonstration that intact chloroplasts are required for *Hpr* expression (Schwartz et al., 1992) and may reflect the need for an adequate rate of phosphoglycolate metabolism during active photosynthesis.

In contrast, the *RbcS* mRNA level increased only 7-fold in response to 24 h of continuous white light (Fig. 2) and less than 4-fold in response to 2-h treatments with white light or red light (Figs. 4 and 8). A 4-h white-light treatment gave twice the *RbcS* response as the 2-h treatment (Fig. 5b), whereas treatments of 1 h or less had no effect on the *RbcS* mRNA level in dark-adapted cotyledons (Fig. 4). This indicates a threshold irradiation time or total fluence requirement of about 2 h or 10^6 μE m^{-2} for an *RbcS* response in dark-adapted cotyledons under our conditions. A total fluence threshold was observed in dark-grown *Arabidopsis* plants for the *RbcS-2B* gene, which responds little to total fluences of from 1 to 1000 μE m^{-2} but markedly to total fluences of 10,000 μE m^{-2} (Dedonder et al., 1993). Similarly, the *RbcS* mRNA level in etiolated pea showed no response to total fluences between 10^{-7} and 0.1 μE m^{-2} s but increased 6-fold above a threshold of 1 μE m^{-2} (Kaufman et al., 1984). Because of the small magnitude of the *RbcS* responses we have observed in dark-adapted cucumber cotyledons, a differentiation between an irradiation time and a total fluence threshold could not be determined.

The *Cab* mRNA level was also less stimulated than that of *Hpr* at irradiations of 1 h or less (Fig. 4), yet it responded to 4 h of irradiation more strongly than did *Hpr* (Figs. 2 and 5a). Maximal stimulation of *Cab* mRNA production appeared to require 2 to 4 h of irradiation, without direct dependence of mRNA level on total fluence. The *Cab* response had two components, showing peaks in mRNA level both at 4 and at 16 to 20 h after irradiation and transfer to darkness, with the level decreasing to near the dark-adapted control levels at 12 h (Figs. 4 and 8). This rapid decline between 4 and 12 h is surprising in light of the stable *Cab* mRNA level seen for 24 h after transfer of plants from continuous white light to darkness (Fig. 1). This difference may reflect more rapid turnover of *Cab* mRNA stimulated by high fluence light as suggested for pea *Cab* mRNA (Marrs and Kaufman, 1991). Alternatively, it may indicate that transcription proceeds for 24 h after transfer from continuous white light to darkness but is more tightly regulated after shorter duration light treatments. Dual peaks were not seen in kinetic studies of *Cab* mRNA accumulation in either dark-adapted soybean suspension cultures (Lam et al., 1989) or etiolated pea seedlings (Kaufman et al., 1986), suggesting either tissue- or species-specific differences. The 16-h spacing between the peaks seems too short to have a circadian basis. It is possible that each of the two cucumber *Cab* genes (Greenland et al., 1987) is responsible for one peak, because the probe used in these experiments did not discriminate between the two.

The *Ubi* gene was originally intended as a light-insensitive control, but we found that the level of its mRNA in cotyledons increased during dark adaptation to approximately twice the continuous white-light level (Fig. 1, a and b insets). In five other experiments (67 samples), the *Ubi* mRNA level in cotyledons of dark-adapted plants was an average of 1.6 ± 0.4 times the level in cotyledons of seedlings maintained in continuous white light (data not shown). Because equal amounts of total RNA were analyzed, this could reflect either a genuine increase in the amount of *Ubi* mRNA in darkness or a decrease in total RNA relative to *Ubi* mRNA. However, its presence regardless of light treatment allows us to conclude that the responses and kinetics of accumulation of the three light-regulated mRNAs were not merely general changes in mRNA abundance of all genes.

The three light-regulated genes differed significantly from each other in their responses to white light or to red light provided by a 600-nm cutoff filter. For both *Hpr* and *RbcS*, filtered red light and unfiltered white light elicited similar responses in cotyledons sampled immediately after irradiation (Fig. 6). For *Cab*, however, irradiation through the red filter reduced the response by about 50%. This finding indirectly suggests a greater dependence on blue-light perception for expression of *Cab* versus *Hpr* or *RbcS* in dark-adapted cucumber cotyledons.

The involvement of phytochrome in *Hpr* expression in dark-adapted cucumber cotyledons is shown by the far-red light-dependent inhibition of white- or red light-stimulated mRNA accumulation (Fig. 7). These results are similar to the findings of Fluhr and Chua (1986) for *RbcS* in mature pea tissue. Because far-red light had little observable effect on *Hpr* mRNA levels in tissue sampled immediately after the light treatments, it appears to inhibit an inductive component of *Hpr* expression. However, the incomplete photoreversibility observed (Fig. 7) and the proportionality of response to irradiation time at a near-saturating fluence rate (Fig. 5) indicate an additional, phytochrome-independent component.

Phytochrome involvement in *Hpr* expression was also shown in etiolated cotyledons using red and far-red LEDs. A 30-min red-LED irradiation of green dark-adapted cotyledons increased the mRNA level only for *Hpr* and a 5-min irradiation affected none of the mRNAs levels, but both treatments increased levels of all three mRNAs in etiolated cotyledons. Five minutes of irradiation with far-red LEDs caused some *Hpr* induction (Fig. 9), probably reflecting the high-irradiance response to far-red light (Mancinelli and Rabino, 1978). The

increase in *Hpr* mRNA level caused by 5-min red-LED treatment was reversed by a subsequent 5-min far-red-LED treatment. Comparison with similar 30-min treatments suggests an escape time for photoreversibility of *Hpr* mRNA accumulation between 5 and 30 min. The results presented here demonstrate conclusively the involvement of phytochrome in regulation of *Hpr* mRNA levels in cucumber cotyledons.

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