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Separate Photosensory Pathways Co-Regulate Blue Light/Ultraviolet-A-Activated *psb*D-*psb*C Transcription and Light-Induced D2 and CP43 Degradation in Barley (*Hordeum vulgare*) Chloroplasts[1]

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We studied the effects of spectral quality and fluence on the expression of several chloroplast-encoded photosynthesis genes and on the stability of their protein products in barley (*Hordeum vulgare*). During light-dependent chloroplast maturation, mRNA levels for *psb*D-*psb*C and *psb*A were maintained at higher levels compared with mRNAs encoding proteins for other photosynthesis functions (*atp*B, *rbc*L). Maintenance of *psb*D-*psb*C mRNA levels was accounted for by differential activation of the *psb*D-*psb*C light-responsive promoter by high-irradiance blue light and, secondarily, ultraviolet A (UV-A) radiation. Promoter activation was fluence dependent and required continuous illumination for 2 h at threshold fluences of 1.3 (blue light), 7.5 (white light), or 10 (UV-A) μmol m^{-2} s^{-1}. From immunoblot analysis experiments, we showed that the *psb*D-*psb*C gene products D2 and CP43 undergo light-mediated turnover similar to light-labile D1. Other photosynthesis proteins such as the β subunit of ATP synthase and the large subunit of ribulose-1,5-bisphosphate carboxylase were relatively stable. In the absence of protein synthesis, D2 degradation paralleled the degradation of D1 (relative half-lives, 9.5–10 h). CP43 decay was about half of D2 and D1 decay. In contrast with activation of the light-responsive promoter, the fluence-dependent degradation of D1, D2, and CP43 required 50- to 100-fold higher fluences of photosynthetically active white, red, blue, or UV-A irradiation. We interpret the different fluence and wavelength requirements to indicate that separate photosensory systems regulate activation of *psb*D-*psb*C transcription and turnover of D1, D2, and CP43. We propose that a blue light/UV-A photosensory pathway activates the *psb*D-*psb*C light-responsive promoter, differentially maintaining the capacity of mature chloroplasts to synthesize D2 and CP43, which are damaged and turned over in illuminated plants.

PSII is one of four multisubunit complexes of thylakoid membranes that catalyze the light reactions of oxygenic photosynthesis. The core of PSII contains seven chloroplast-encoded polypeptides (Mattoo et al., 1989; Vermaas and Ikeuchi, 1991). These proteins and their genes are D1 (*psb*A), D2 (*psb*D), CP47 (*psb*B), CP43 (*psb*C), Cyt b_{559} (*psb*E/F), and subunit PSII-I (*psb*I). The homologous subunits D1 and D2 associate to form a heterodimer that binds between 4 to 10 Chls, 2 pheophytins, 2 quinones (Q_A, Q_B), Fe^{2+}, and Mn^{2+}. CP43 and CP47 represent Chl proteins that function as core light-harvesting antennae. Several studies have documented that D1 undergoes rapid light-induced turnover (reviewed by Mattoo et al., 1989). In *Chlamydomonas*, light-induced degradation of D2 has been reported (Schuster et al., 1988). The turnover of D1 and D2 is a consequence of PSII photochemistry (Greenberg et al., 1989; Barber and Andersson, 1992). Their loss from PSII could block noncyclic electron transport and destabilize the remaining PSII components, as observed in D1- and D2-deficient mutants (Vermaas and Ikeuchi, 1991).

Light plays a pivotal role in regulating the synthesis, accumulation, stoichiometry, and turnover of the protein constituents of PSII (Anderson, 1986; Mullet, 1988; Kim et al., 1993a). In higher plants, the effects of light are mediated by three major photoreceptors: the phytochromes (Quail, 1991), the Pchlide holochrome (Thompson and White, 1991), and the blue light/UV-A photoreceptors (Kaufman, 1993). Red light acting through phytochrome modulates chloroplast development (Mullet, 1988) and the expression of several chloroplast (Thompson et al., 1983) and nuclear-encoded photosynthesis genes. The latter include genes encoding the Chl *a*/*b*-binding proteins (*Lhcb*) of the light-harvesting complex, the small subunit (*Rbcs*) of Rubisco, and Pchlide reductase (Fluhr and Chua, 1986; Thompson and White, 1991), an enzyme involved in Chl biosynthesis. Pchlide also absorbs red light and is converted to Chlide, a precursor to Chl. Because Chl is required for the stable accumulation of the Chl apoproteins (Klein et al., 1988; Mullet et al., 1990), it is essential for PSII assembly.

The role of blue light/UV-A in PSII biogenesis is less clear. Blue light regulates the expression of nuclear-encoded *Lhcb* (Kaufman, 1993) and *Rbcs* (Fluhr and Chua, 1986) genes and, in chloroplasts, a special class of *psb*D-*psb*C mRNAs (Gamble and Mullet, 1989b). Recently, we have shown that these blue-light-induced *psb*D-*psb*C mRNAs arise by light-induced transcription from a new type of chloroplast promoter, designated the *psb*D-*psb*C light-responsive promoter (Sexton et al., 1990; Christopher et al., 1992). Because light-regulated

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Abbreviations: ATPβ, β subunit of ATP synthase; CP43 and CP47, chlorophyll proteins of the core light-harvesting antennae of PSII; D1 and D2, core subunits of the PSII reaction center; LS Rubisco, large subunit of ribulose-1,5-bisphosphate carboxylase.

*psb*D-*psb*C expression is phylogenetically conserved in higher plants (Christopher et al., 1992) and cyanobacteria (Bustos and Golden, 1992), it may play an important functional role in photosynthetic organisms.

The function of blue light-regulated *psb*D-*psb*C expression could be related to the light-induced turnover of D2 and CP43. D1 appears to be particularly unstable under blue light and UV radiation, which are proposed to preferentially excite bound quinone (Greenberg et al., 1989). The instability of D1 and D2 under high light places a complex physiological and regulatory demand on plants. If photodegraded subunits are not continuously replaced, PSII activity decreases (Barber and Andersson, 1992). This is further complicated by the fact that, as chloroplasts mature, overall plastid transcription, mRNA levels, and translation decline (Mullet, 1988). Therefore, to prevent loss of PSII activity, special regulatory mechanisms are necessary to differentially sustain the production of D1 and D2 in mature chloroplasts, especially when plants are exposed to high light.

Recently, we proposed that blue-light activation of *psb*D-*psb*C transcription could be a regulatory mechanism that enhances the rates of D2 and CP43 synthesis, thus helping to maintain PSII activity under high light (Christopher et al., 1992). However, the in vivo turnover of D2, especially compared with D1 turnover, has not been studied in plants known to have light-induced *psb*D-*psb*C expression. Also, the relationship between light-regulated photosynthesis gene expression and light-mediated PSII subunit degradation is not well understood. Therefore, in this report, we examine the effects of spectral quality and fluence on the expression of several chloroplast-encoded photosynthesis genes and on the turnover of their protein products. We report that separate photosensory pathways regulate activation of *psb*D-*psb*C transcription and light-dependent degradation of the PSII subunits D1, D2, and CP43. We propose that a blue light/UV-A photosensory pathway activates *psb*D-*psb*C expression, providing the capacity to synthesize and replace D2 and CP43, which are turned over in mature chloroplasts. In this model, a blue-light signaling pathway is believed to play a significant role in maintaining PSII activity.

MATERIALS AND METHODS

Plant Growth and Plastid Isolation

Barley (*Hordeum vulgare* L. cv Morex) seeds were sterilized in 20% household bleach with 0.2% Tween 20 for 5 min and washed in sterile water. Seeds were then allowed to imbibe for 2 h and planted in flats onto two sheets of gel-blotting paper (grade GB003, Schleicher & Schuell) that was layered over vermiculite saturated with half-strength Hoagland nutrient solution. Seedlings were grown at 23°C in a light-tight, controlled environment chamber for 3.5, 4, and 4.5 d. The dark-grown seedlings were either harvested under a dim-green safelight or treated in one of the following ways: (a) illuminated with white, blue, or red light or UV-A or UV-B radiation for 2 h as described below or (b) exposed to continuous white light (fluorescent plus incandescent bulbs at 75 or 350 μmol m^{-2} s^{-1}) for an additional 2, 3, or 4 d before harvesting or further use in the in vivo chloramphenicol uptake and light experiments described in this paper.

Plastids were isolated from the top 4 cm of barley primary leaves by centrifugation of cell lysates on Percoll gradients (40–80%) as described (Klein and Mullet, 1987). Intact plastids were quantitated (plastids per microliter) by counting a dilute suspension in a hemacytometer.

RNA Analysis

Plastid and total cell RNAs were isolated by buffered organic extraction as previously described (Christopher et al., 1992). Plastid RNAs (see Fig. 1) were resuspended and used in further experiments on an equal plastid basis, whereas total RNAs (see Fig. 2) were used on an equal RNA basis. Techniques for barley RNA gel-blot analyses were as described by Sexton et al. (1990). Linearized recombinant plasmids contained DNA inserts specific for *rbc*L, *psb*A, and *atp*B as reported by Rapp et al. (1992). Gene-specific antisense RNA probes were synthesized and radiolabeled with [α-^{32}P]-UTP using T3 and T7 RNA polymerases. The deoxyoligonucleotide 5′-GATTCAGCATTATTCCAG was labeled at the 5′ end and used in primer extension analysis as described (Christopher et al., 1992).

Light and Radiation Sources

Photon fluences (μmol m^{-2} s^{-1}) for red, blue, and white light were measured using a quantum photometer (LI-185B, Li-Cor, Inc., Lincoln, NE). Fluence rates for UV-A and UV-B radiation were measured using a J-221 UV meter (Ultra Lum, Inc., Carson, CA). Fluence rates were varied by changing the distance between the plants and irradiation sources and by using layers of Miracloth (Calbiochem) as neutral density filters. All manipulations of dark-grown plants were performed in either complete darkness or under a dim-green safelight (Klein et al., 1988). Light experiments were conducted in a specially constructed light-tight chamber in a darkroom. The following sources of irradiation were used:

(a) Red light (0.3–75 μmol m^{-2} s^{-1}) above 600 nm was obtained by passing fluorescent light (F40T12/CW or F15T8/D, Sylvania) through two red filters (No. 2423, 3-mm thick Plexiglas, A-1 Plastics Co., Austin, TX) that eliminate all light below 600 nm. Red light at 250 μmol m^{-2} s^{-1} was obtained by passing incandescent light (200 W soft white or 50 W R-20 flood light, General Electric) though a clear, tempered-glass plate, a 2-cm layer of water (cooled with portable ice packets), and double and single red Plexiglas cut-off filters (No. 2423). Additional cooling of the apparatus was obtained by circulating cool air provided by small fans and hair dryers.

(b) Blue light (0.3–25 μmol m^{-2} s^{-1}) was obtained by passing fluorescent light (F20T12/Blue, F15T8/D, Sylvania) through single and double blue Plexiglas filters (No. 2424 for 385–535 nm, peak 470 nm, and No. 2051 for 402–530 nm, peak 462 nm, A-1 Plastics). Similar levels of *psb*D-*psb*C mRNA accumulation were observed using each type of filter. Blue light (75 and 220 μmol m^{-2} s^{-1}) was obtained by passing incandescent light (200 W soft white, General Electric) through single and double blue (No. 2424) filters that were cooled with fans as above.

(c) White light (0.3–75 μmol m^{-2} s^{-1}) was obtained from fluorescent light lamps (F15T8/D, Sylvania) in a light-tight

chamber. Higher-intensity white light at 350 and 750 μmol m^{-2} s^{-1} was obtained from fluorescent (F72T12/CW/HO, Sylvania) plus incandescent bulbs (60 W, Westinghouse) in a GC-15 Environmental Growth Chamber (Chagrin Falls, OH).

(d) UV-A radiation (0.3–75 and 380 μmol m^{-2} s^{-1}) was obtained using self-filtering, black-light blue bulbs (F15T8/BLB, Sylvania), which absorb virtually all visible light. The spectral output of the F15 lamp, as described by the manufacturer (Sylvania engineering bulletin No. O-306), is in the range of 300 to 405 nm with a peak at 375 nm.

(e) UV-B radiation from 290 to 340 nm was obtained using a self-filtering lamp (model UVB-16, Ultra-Lum).

(f) Far-red light was obtained as described by Gamble and Mullet (1989b).

In Vivo Chloramphenicol Uptake and Protein Decay Experiments

Seedlings grown for 3.5 and 4 d in darkness followed by 2 d in light (either 75 or 350 μmol m^{-2} s^{-1}, described above) were excised at the point of attachment to the seed. A total of 80 seedlings per treatment were placed in a small beaker containing a solution of 0.5% Suc plus 0.5× Murashige and Skoog basal salts (Sigma) with or without 300 μg/mL chloramphenicol. The seedlings were maintained in these solutions for 2 h in white light (75 μmol m^{-2} s^{-1}). Seedlings were then either harvested immediately after chloramphenicol treatment (0 h) or transferred to darkness or to one of the following light regimes: white (75, 350, or 750 μmol m^{-2} s^{-1}), blue (25 or 220 μmol m^{-2} s^{-1}), or red (25 or 250 μmol m^{-2} s^{-1}) light or UV-A radiation (380 μmol m^{-2} s^{-1}) for a 5-, 10-, 20-, or 25-h period before harvesting and plastid isolation. When necessary, the beaker was supplemented with fresh solution with or without 100 μg/mL chloramphenicol. Chloramphenicol treatments were also conducted in parallel on intact barley seedlings (roots attached) grown in tall, glass Petri dishes (100 × 80 mm, Corning, Inc., Corning, NY). Protein degradation rates of intact seedlings occurred within the range of, but were not as consistent as, those obtained with excised seedlings. Hence, data from the excised seedlings are reported here.

Immunological Analysis of Chloroplast Proteins

In each experiment, chloroplast proteins were analyzed on an equal plastid basis. Chloroplasts (2.5×10^6) were fractionated into soluble and membrane phases as described (Mullet et al., 1986), dissolved in loading buffer (50 mM Tris-HCl pH, 6.8, 2% SDS, 70 mM DTT, 10% glycerol, and 0.05% bromphenol blue), and heated in a boiling-water bath for 3 min. Denatured proteins were then separated on 12.5% polyacrylamide (acrylamide:bis, 37.5:1, w/w) gels containing 4 M urea and 0.1% SDS. After electrophoresis, chloroplast proteins were visualized by Coomassie staining (0.1%) or were electrophoretically transferred to nitrocellulose overnight (35 V, 4°C) in a buffer of 25 mM Tris-HCl, pH 8.3, 96 mM Gly, 15% methanol.

The preparation of rabbit antisera specific for the D1, D2, and CP43 polypeptides as *trp*E fusion proteins was previously described (Gamble and Mullet, 1989a). Antisera generated against ATPβ was a generous gift of Dr. Nam-Hai Chua. For immunodetection of chloroplast proteins immobilized on nitrocellulose, the optimal dilutions of each antiserum were initially determined. These dilutions were as follows: D1 (1:1000), D2 (1:3000), CP43 (1:5000), ATPβ (1:3000). The protein gel blots were blocked with incubation solution (5% Carnation nonfat milk, 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20). Antisera were diluted with incubation solution, added to the blots, and incubated for 1 h at 23°C with gentle shaking. Blots were washed with four changes of wash solution (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) and then incubated for 1 h with commercial anti-rabbit secondary antibody (donkey IgG) that was conjugated to horseradish peroxidase (diluted 10^{-4}, Amersham). After washing as above, antibody:antigen complexes were visualized with a chemiluminescent substrate (Amersham) and autoradiography. The same blots were stripped (in 100 mM 2-mercaptoethanol, 1% SDS, 65 mM Tris-HCl, pH 6.8) at 45°C for 30 min and probed again consecutively with each of the different antisera.

Quantitation of Signals and Bands

Radioactive ^{32}P signals on RNA gel blots and dried polyacrylamide gels were quantitated using a Betascope Blot Analyzer (Betagen Co., Framingham, MA). Bands present on autoradiograms were quantitated by using a Visage 110–20 densitometer (Bioimage, Inc., Rochester, NY).

RESULTS

Differential Maintenance of *psb*D-*psb*C and *psb*A mRNAs in Mature Chloroplasts

In Figure 1 we compared the levels of mRNAs for *psb*D-*psb*C, *psb*A, *atp*B, and *rbc*L using primer extension analysis and RNA gel-blot experiments. Plastid RNAs from seedlings representing three different stages of chloroplast development were analyzed: 4-d-old, dark-grown seedlings (4 DD); 4 DD plus 2 d of continuous light (2 DL); and 4 DD plus 4 d of continuous light (4 DL). The levels of the light-induced *psb*D-*psb*C mRNAs (RNAs marked with an arrow) remain relatively high at 4 DL (Fig. 1A). In contrast, the higher mol wt *psb*D-*psb*C mRNAs and *rbc*L and *atp*B mRNAs decrease sharply during chloroplast development.

Total *psb*D-*psb*C, *rbc*L, and *psb*A mRNA levels were quantitated and these values are graphed in Figure 1B. After 2 d of light (2 DL), the monocistronic mRNAs for *atp*B and *rbc*L decreased to half of the level present in 4 DD seedlings. In contrast, after 2 d of light, the mRNAs for *psb*A and *psb*D-*psb*C were 90% of 4 DD levels. By 4 d of light, *atp*B and *rbc*L mRNAs decreased to less than 10% of the levels in 4 DD seedlings, whereas *psb*A and *psb*D-*psb*C decreased to 50%. In this experiment, we show that mRNAs encoding core components of PSII (D1, D2, and CP43) are differentially maintained in mature chloroplasts compared with mRNAs (*atp*B, *rbc*L) for other photosynthetic functions. Specifically, the transcripts that arise from the *psb*D-*psb*C light-responsive promoter (Christopher et al., 1992) account for the maintenance of *psb*D-*psb*C mRNA levels during light-dependent chloroplast maturation.

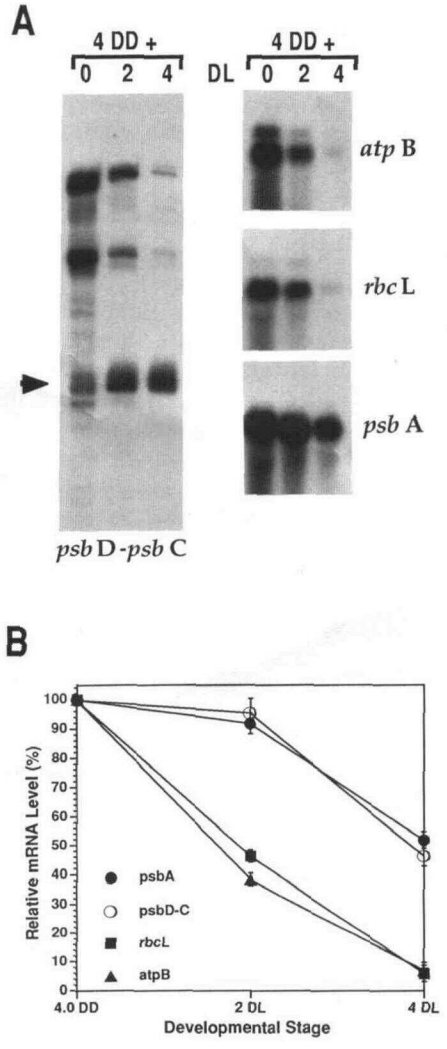

Figure 1. Analysis of chloroplast photosynthesis gene expression during three stages of chloroplast development. A, *psb*D-*psb*C mRNAs were analyzed by primer extension and *atp*B, *rbc*L, and *psb*A mRNAs were analyzed by RNA gel-blot hybridization on an equal plastid basis. Plastids were isolated from barley seedlings grown for 4 d in darkness (4 DD) and transferred to either 2 d (2 DL) or 4 d (4 DL) of continuous white light (350 μmol m^{-2} s^{-1}). The black arrowhead points to the light-induced *psb*D-*psb*C mRNAs. B, The total counts in each lane were quantitated from duplicate experiments as shown in A and plotted for each developmental stage.

Regulation of *psb*D-*psb*C by High-Irradiance Blue Light and UV-A

Experiments were conducted to obtain a better understanding of the photosensory pathways involved in the regulation of *psb*D-*psb*C expression. The experiments shown in Figure 2 distinguish between the effects of fluence and spectral quality. Barley seedlings were grown for 4.5 d in darkness and then transferred to one of six different fluences ranging from 0.3 to 75 μmol m^{-2} s^{-1} of continuous white, blue, red, UV-A, or UV-B irradiation for a 2-h period. The effects of individual light treatments on *psb*D-*psb*C mRNA accumula-

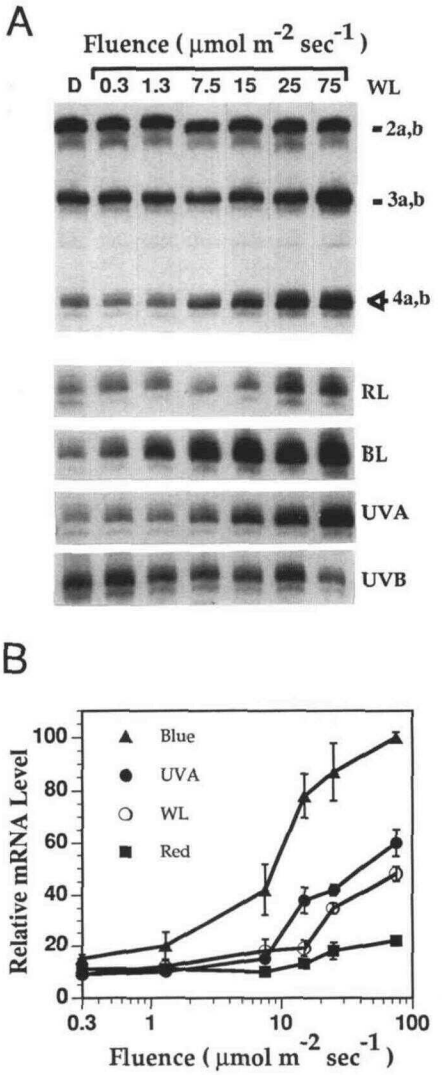

Figure 2. Effect of spectral quality and fluence on *psb*D-*psb*C expression in barley seedlings. A, *psb*D-*psb*C mRNAs were assayed by primer extension analysis. Total RNAs were isolated from 4.5-d-old, dark-grown seedlings that were transferred to 2 h of continuous illumination consisting of white (WL), red (RL), or blue light (BL) or UV-A or UV-B radiation. Fluences incident at barley leaf tips are indicated in μmol m^{-2} s^{-1} at the top of each lane as 0.3, 1.3, 7.5, 15, 25, and 75. The D above the left lane denotes dark-grown control. In the top panel, an open arrowhead points to the light-induced *psb*D-*psb*C mRNAs. The 2a,b and 3a,b designate *psb*D-*psb*C mRNAs present in dark-grown plants. Only the light-induced mRNAs are shown for the remaining treatments. To visualize bands, the UV-B gel was overexposed compared with gels for the other treatments. B, Quantitation of the accumulation of the light-induced *psb*D-*psb*C mRNAs under changing light environments. Relative mRNA levels were determined by quantitating radioactivity in bands corresponding to specific mRNAs from experiments shown in A. To correct for possible loading errors, the values for the light-induced mRNAs were normalized by dividing by the values for mRNAs 2a,b, which did not change during the 2-h illumination. The highest mRNA level was set at 100. Normalized values (scale 0–100) were plotted with respect to fluence rate. Experiments were done in duplicate, except those for blue and red light, which were done in triplicate.

tion were assayed by primer extension analysis (Fig. 2A) and quantitated (Fig. 2B).

A fluence-dependent accumulation of the light-induced *psb*D-*psb*C mRNAs was observed in white light (Fig. 2A, upper panel, RNAs marked with an arrow) with a fluence requirement of 7.5 μmol m^{-2} s^{-1} for 2 h. This fluence dependence was also observed for blue light lacking UV-A radiation. Blue light produced the greatest increase in *psb*D-*psb*C mRNAs. A blue-light fluence of 1.3 μmol m^{-2} s^{-1} was necessary to detect an increase over dark controls after 2 h of treatment. This fluence was less than one-tenth the fluence of white light that gave a comparable induction. UV-A radiation elicited a response that was approximately 50% of the blue-light effect. In striking contrast, very little induction was obtained with red light (Fig. 2A, RL). No effects of UV-B radiation on the induction of *psb*D-*psb*C mRNAs were observed. These experiments established that blue light and UV-A were more effective inducers of *psb*D-*psb*C transcription than red light or UV-B.

To examine the involvement of phytochrome in blue-light-induced *psb*D-*psb*C transcription, we tested the ability of continuous far-red light to prevent the accumulation of blue-light-induced *psb*D-*psb*C mRNAs. As shown in Table I, both continuous blue light and alternating blue light/dark were effective inducers of *psb*D-*psb*C transcription. However, continuous far-red light given together with 5-min pulses of blue light had no significant effect on blue-light-induced *psb*D-*psb*C transcription. Therefore, the accumulation of blue-light-induced *psb*D-*psb*C mRNAs was not controlled primarily by blue light acting through phytochrome.

As shown in Figure 3, the effects of 2 h of blue, red, or UV-A irradiation on *rbc*L and *psb*A mRNA levels were quantitated as described for *psb*D-*psb*C. Two fluences that induced *psb*D-*psb*C mRNA levels were utilized. No new *psb*A and *rbc*L mRNAs were induced in these light treatments. For both genes, however, an overall light-induced increase in their mRNA levels was detected, but this increase was not specific to blue, red, or UV-A irradiation. Only *psb*D-*psb*C showed selective regulation by blue light. Therefore, blue light and, secondarily, UV-A radiation appear to be the most effective photosensitizers activating the light-regulated *psb*D-*psb*C promoter. The blue-light regulation results in the maintenance of *psb*D-*psb*C mRNA levels as chloroplasts mature (Figs. 1 and 2).

Table I. *Effect of far-red light on the blue-light induction of the psbD-psbC light responsive promoter*

Dark-grown seedlings (Dark) (4.5 d) were exposed to 3 h of continuous blue light and 5 μmol m^{-2} s^{-1} (Continuous blue light-5) or were exposed to 5 min of blue light at 5 μmol m^{-2} s^{-1} followed by 5 min of darkness for 3 h (Alternating blue light/dark). Alternating blue light/dark was also given together with continuous far-red light at 5 μmol m^{-2} s^{-1} for 3 h. *psb*D-*psb*C expression was assayed by primer extension analysis. The amount of radioactivity (cpm) present in primer extension products was quantitated for three replicates of one experiment. Relative mRNA levels are also represented as a percentage of the highest value (100%), which was for continuous blue light.

Treatment	Relative mRNA Level	
	cpm ± *SE*	%
Dark	6.5 ± 5.3	11%
Continuous blue light-5	59.3 ± 11.5	100%
Alternating blue light/dark	47.0 ± 4.1	79%
Alternating blue light/dark + continuous far-red	40.1 ± 6.6	68%

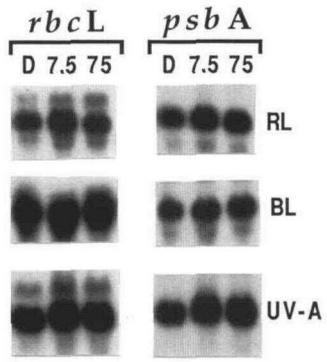

Figure 3. Effect of spectral quality and fluence on *rbc*L and *psb*A mRNA levels. RNA samples (as described in Fig. 2) were analyzed in RNA gel-blot hybridization experiments using gene-specific probes. The treatments were 4.5-d-old, dark-grown barley (D), plus 2 h of red (RL) or blue light (BL) or UV-A radiation (UV-A) at fluence rates of either 7.5 or 75 μmol m^{-2} s^{-1}.

Immunoblot Analysis of Light-Mediated and Fluence-Dependent Degradation of D2 and CP43

The blue-light-induced accumulation of *psb*D-*psb*C mRNAs was next related to the effects of light on the stability of the *psb*D-*psb*C gene products D2 and CP43. The levels of these proteins were followed under different light-intensity conditions in the presence of an inhibitor of plastid protein synthesis, chloramphenicol. Seedlings were grown for 3.5 d in darkness and for 2 d in light prior to chloramphenicol treatments. Antisera specific for D2 and CP43 were utilized for immunodetection. Representative results are shown in Figure 4. Very little decrease in D2 and CP43 was observed when plants were exposed to 75 μmol m^{-2} s^{-1} of white light for 20 h in the presence of chloramphenicol (Fig. 4). However, increasing the fluence to 350 and 750 μmol m^{-2} s^{-1} produced a sharp decrease in the levels of these proteins per chloroplast. The decrease in D2 was greater than that of CP43, and the decrease of both proteins was fluence dependent.

Because the turnover of these polypeptides was minor when plants were treated with 75 μmol m^{-2} s^{-1} of white light plus chloramphenicol, we concluded that secondary effects of this inhibitor did not contribute to protein turnover and that the decay was caused by the higher-intensity light (350 μmol m^{-2} s^{-1}). Because no protein decline was observed in the minus-chloramphenicol controls, we conclude that plants normally maintain protein levels through increased rates of protein synthesis when high light causes an increase in protein turnover. Recognizing the possible complications of using chloramphenicol, we initially used in vivo pulse/chase assays with [^{35}S]Met to examine D1, D2, and CP43 turnover. However, the in vivo pulse/chase assays of barley seedlings proved problematic.

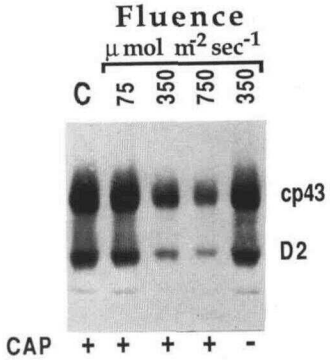

Figure 4. Immunoblot analysis of the effect of fluence rate on PSII subunit degradation. Seedlings grown for 3.5 d in darkness and 2 d in light (350 μmol m^{-2} s^{-1}) were treated with (+) or without (−) chloramphenicol (CAP) as described in "Materials and Methods." Seedlings were then returned to 20 h of continuous white light at 75, 350, or 750 μmol m^{-2} s^{-1}. The C refers to control, plus CAP, but prior to the 20-h light treatment (time 0). Thylakoid membranes from an equal number of purified chloroplasts were separated by SDS-PAGE and transferred to nitrocellulose. Protein blots were hybridized with anti-CP43- and anti-D2-specific antisera and signals were visualized using a chemiluminescent substrate.

For a more detailed analysis of D2 and CP43 turnover under high light, we used a fluence rate of 350 μmol m^{-2} s^{-1}. Seedlings were pretreated with chloramphenicol and placed in either darkness or light as described in "Materials and Methods." The levels of D2 and CP43 and three other chloroplast-encoded polypeptides involved in photosynthesis, ATPβ encoded by *atp*B, the LS Rubisco encoded by *rbc*L, and PSII polypeptide D1 encoded by *psb*A were followed over a 25-h time course. D1 was analyzed because light-induced turnover of this protein has been well documented (Mattoo et al., 1984). Both LS Rubisco and ATPβ served as additional controls for soluble and membrane-associated chloroplast proteins, respectively, that are involved in different photosynthetic functions.

Immunoblots are shown in Figure 5A. A comparison of polypeptide levels from replicated experiments is presented in Figure 5B. When light-grown plants were treated with chloramphenicol and transferred to darkness, very little change in polypeptide levels occurred for 25 h (Fig. 5A). However, when plants were maintained in 350 μmol m^{-2} s^{-1} white light for up to 25 h, a significant light-mediated decrease (approximately 80%) in the levels of D1 and D2 per chloroplast was observed (Fig. 5, A and B). This decrease was clearly evident by 10 h. The levels of CP43 decreased less, approximately 45%, during the 25-h period. The levels of ATPβ and LS Rubisco did not change substantially when chloramphenicol-treated seedlings were exposed to darkness or light. The slight decrease in LS Rubisco was not light specific. Other thylakoid polypeptides (PSI P700 apoproteins, light-harvesting complex) that were followed by Coomassie staining did not decrease in abundance in illuminated plants (data not shown). Thus, under conditions in which barley chloroplast protein synthesis is inhibited, a significant light-dependent decrease in the levels of PSII polypeptides D1, D2, and CP43 occurred relative to other photosynthetic proteins. The turnover rates of D2 and D1 were similar (Fig. 5B).

Effect of Blue, Red, and UV-A Irradiation of PSII Polypeptide Decay

Because the activation of *psb*D-*psb*C is regulated by blue light and because the gene products show light-mediated degradation, it was of interest to determine the effect of light quality on protein degradation. Protein turnover experiments using immunoblots similar to those shown in Figure 5 were

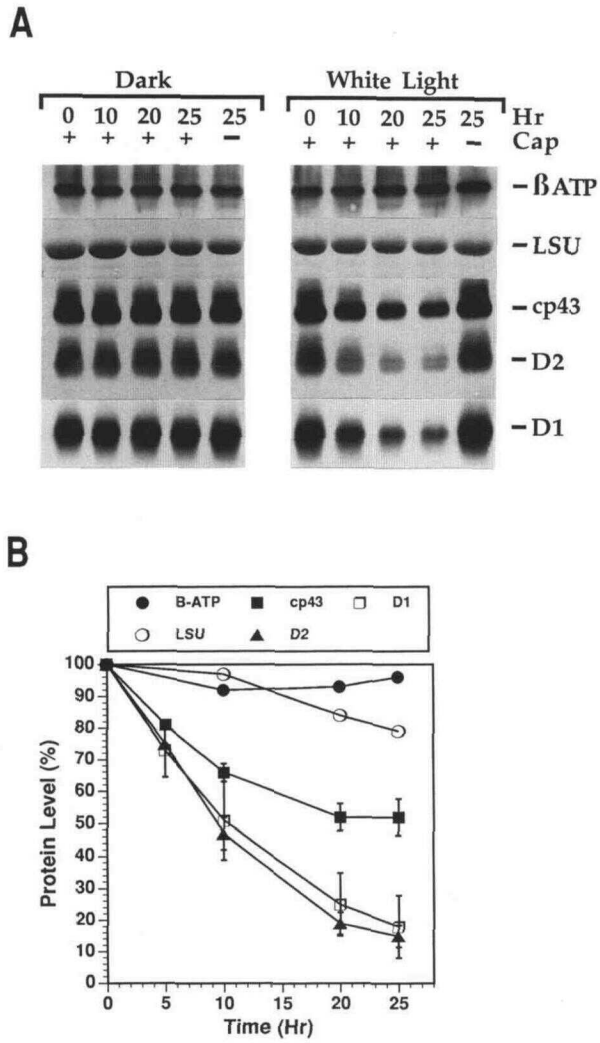

Figure 5. Immunoblot analysis of the time course for photosynthetic polypeptide degradation under constant dark or white light (350 μmol m^{-2} s^{-1}) conditions. A, Three-and-one-half-day-old, dark-grown barley seedlings were transferred to 2 d of continuous illumination (350 μmol m^{-2} s^{-1}) prior to chloramphenicol (Cap) treatment (see "Materials and Methods"). Seedlings were then transferred to darkness or white light (350 μmol m^{-2} s^{-1}) for 0, 10, 20, or 25 h with (+) or without (−) Cap. Purified chloroplasts were fractionated into membrane and soluble phases on an equal plastid basis and separated by the use of SDS-PAGE. Immunoblots were hybridized with antisera specific for ATPβ and PSII subunits CP43, D2, and D1. After stripping, the same blot was then hybridized again with a different antibody. The LS Rubisco was analyzed by Coomassie staining. B, The relative values of bands present in duplicate experiments as described in A were quantitated by densitometry and plotted with respect to time.

conducted. After the chloramphenicol pretreatment, white-light-grown seedlings were transferred either to the dark or to white, blue, red, or UV-A irradiation. For each light treatment, the levels of D2, CP43, D1, and, as controls, ATPβ and LS Rubisco were measured during a 25-h time course.

The effects of irradiation wavelength on the turnover of the D2, CP43, and D1 proteins are presented in Figure 6. Regardless of wavelength, each polypeptide was degraded in a light-dependent manner. However, differences were evident in the effectiveness of each type of irradiation on protein turnover. For each polypeptide, consistently more rapid and, generally, greater protein degradation occurred under blue light (220 μmol m^{-2} s^{-1}) than under red light (250 μmol m^{-2} s^{-1}). For example, 10 h of blue light caused D2 and D1 levels to decrease by at least 50%, whereas the levels of these proteins after 10 h of red light remained relatively high (87–90%). After 20 h of blue light, the levels of D1 and D2 proteins decreased to less than 30%, whereas about 50% of their levels remained after 20 h of red light. Blue light also had a greater effect than red light on the turnover of the CP43 protein. Of the three PSII polypeptides studied, CP43 was the least susceptible to light-induced turnover. Overall, blue light (220 μmol m^{-2} s^{-1}) more closely paralleled the effects of white light (350 μmol m^{-2} s^{-1}).

In visible light, the degradation rates of D2 resembled those of D1. In contrast, under UV-A radiation (380 μmol m^{-2} s^{-1}), D1 was degraded more rapidly than D2. After 10 h of UV-A irradiation, approximately 60% of the D1 protein had degraded compared with controls. In comparison, after 10 h of UV-A, the levels of D2 protein were reduced 32%. After 25 h of irradiation, differences in spectral sensitivity of each protein and the degree of wavelength-specific effects had diminished.

The fluences used in the experiments presented in Figure 6 were much higher than the fluences required for *psb*D-*psb*C promoter activation (Fig. 2). It was of interest to determine whether the fluences required for gene induction would also cause protein degradation. Consequently, a blue-light fluence that saturated gene induction (25 μmol m^{-2} s^{-1}) was tested for its effects on protein degradation. The effects of blue light were compared with those of red light (25 μmol m^{-2} s^{-1}) and to those of white, red, blue, and UV-A irradiation at higher fluences. The levels of PSII polypeptides that remained after the 20-h treatment period are summarized in Figure 7.

After 20 h of 25 μmol m^{-2} s^{-1} blue or red light, the levels of each polypeptide examined remained relatively unchanged compared with the significant decreases obtained with 10-fold higher fluence rates (Fig. 7). For D1 and D2, the effects of blue (220 μmol m^{-2} s^{-1}) and UV-A (380 μmol m^{-2} s^{-1}) were similar. Differences in lability of D2 and D1 can be summarized in the relative order of effectiveness of each light source for causing protein decay. For D2 degradation, the relative order of effectiveness was blue (220 μmol m^{-2} s^{-1}) and UV-A (380 μmol m^{-2} s^{-1}) > red (250 μmol m^{-2} s^{-1}) > lower-fluence red and blue (25 μmol m^{-2} s^{-1}). For D1 the relative order was UV-A (380 μmol m^{-2} s^{-1}) and blue (220 μmol m^{-2} s^{-1}) > red (250 μmol m^{-2} s^{-1}) > lower-fluence red and blue (25 μmol m^{-2} s^{-1}). Therefore, the fluences that activate gene expression are relatively ineffective inducers of photodamage of D2 and CP43. However, the differences between the effectiveness of red and blue light are consistent, indicating that the degradation of the D2 and CP43 proteins are more photosensitive to blue/UV-A wavelengths.

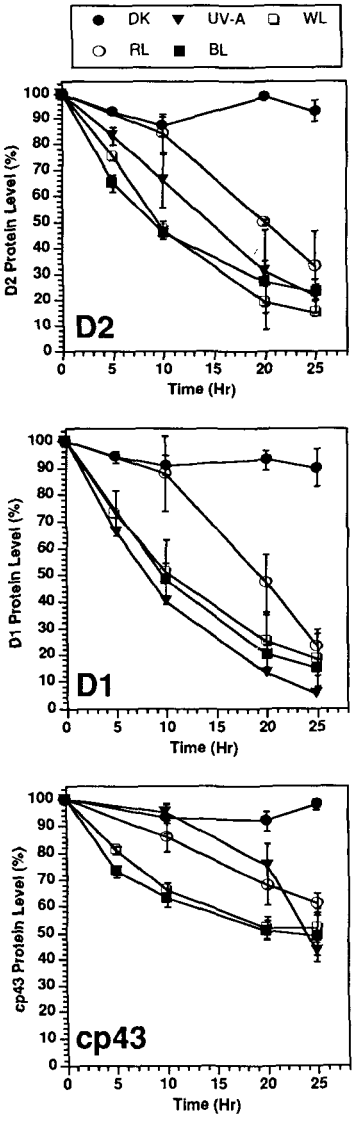

Figure 6. Effect of spectral quality on the degradation of PSII subunits D2, D1, and CP43. The experimental set-up was exactly as described in Figure 5 except that after chloramphenicol treatment, seedlings were transferred to 250 ± 10 μmol m^{-2} s^{-1} red (RL), 220 ± 10 μmol m^{-2} s^{-1} blue (BL), 350 ± 10 μmol m^{-2} s^{-1} (WL), 380 ± 20 μmol m^{-2} s^{-1} (UV-A), or darkness (DK). Immunoblots were used to analyze the levels of each polypeptide during the time course. Bands from duplicate experiments were quantitated and normalized by dividing the values for D2, D1, and CP43 by the values for ATPβ.

DISCUSSION

We studied the relationship between light-regulated expression of photosynthesis genes and light-dependent turnover of the proteins encoded by those genes. The results are relevant to understanding how plant cells maintain PSII activity under changing conditions of light quality and inten-

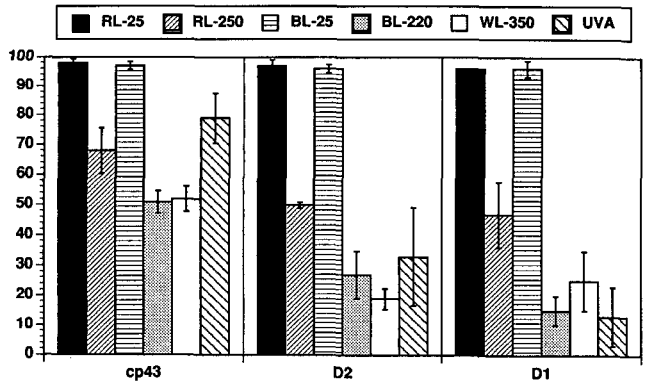

Figure 7. Comparison of the effects of spectral quality and fluence on the degradation of PSII subunits D2, D1, and CP43. Subunits were analyzed on immunoblots as described in Figure 6. The amount of D2, D1, and CP43 remaining after 20 h is shown. RL-25 and RL-250 refer to red light at 25 and 250 μmol m^{-2} s^{-1}. BL-25 and BL-220 refer to blue light at 25 and 220 μmol m^{-2} s^{-1}. The intensity of white light (WL-350) was 350 μmol m^{-2} s^{-1}, and UV-A was 380 μmol m^{-2} s^{-1}.

sity. We show that the photosensory pathway mediating blue-light/UV-A activation of *psb*D-*psb*C transcription is independent of light-mediated degradation and turnover of the gene products D2 and CP43. Gene activation occurs by way of a blue-light/UV-A signaling system, whereas protein degradation is regulated by different photosensitizers, probably plastoquinone and Chl, which require 50- to 100-fold higher fluences of red, blue, or UV-A radiation. Although gene activation and gene product degradation are uncoupled in terms of photobiological requirements, the two separate processes are co-regulated by light and have probably co-evolved as part of a mechanism to sustain PSII function in changing light environments.

A Photosensory Pathway for High-Fluence Blue Light/ UV-A Activates a Chloroplast *psb*D-*psb*C Promoter

In earlier work, the accumulation of the light-induced *psb*D-*psb*C mRNAs was shown to be regulated by blue light (Gamble and Mullet, 1989b) at the transcriptional level from a novel light-responsive *psb*D-*psb*C promoter (Christopher et al., 1992). In this paper, we distinguished between the effects of blue, UV-A, and UV-B irradiation. We have shown that high-fluence blue light and, secondarily, UV-A are the principal wavelengths involved in the differential activation of the promoter. To our knowledge, this is the only example described to date of a chloroplast promoter regulated by blue light/UV-A.

The work described here further supports the conclusion (Gamble and Mullet, 1989b) that light regulation of *psb*D-*psb*C is a high-irradiance response. Activation of the blue-light-responsive promoter occurred in a fluence-dependent manner. The threshold fluence for blue light was 1.3 μmol m^{-2} s^{-1} given continuously for 2 h (approximately 1×10^{-4} μmol m^{-2}). Near saturation was reached at the fluences of 15 to 25 μmol m^{-2} s^{-1} for 2 h (approximately 1 to 2×10^{-5} μmol m^{-2}) in agreement with Gamble and Mullet (1989b). Moreover, it was previously shown that accumulation of the blue-light-induced *psb*D-*psb*C mRNAs can be partially modulated by pulses of far-red light, whereas continuous far-red light alone was noninductive (Gamble and Mullet, 1989b). From quantitative experiments reported here (Table I), continuous far-red light had little effect in reducing blue-light-induced *psb*D-*psb*C transcription. Hence, the involvement of blue light and UV-A radiation is similar to that of other high-irradiance blue-light-regulated processes in plants that have peaks in both blue and UV-A regions of the spectrum (Mancinelli and Rabino, 1978). Likewise, the blue-light fluences required for *psb*D-*psb*C activation closely resemble the high irradiances required for blue-light-mediated hypocotyl inhibition in *Arabidopsis* (Liscum and Hangarter, 1991). In contrast, the total blue-light fluences and exposure times required for activating nuclear gene (*Lhca/b*) transcription (Marrs and Kaufman, 1989) and a G-protein (Warpeha et al., 1991) and membrane protein kinase (Reymond et al., 1992; Short et al., 1992) are at least 1 order of magnitude lower than the fluences required for activating the *psb*D-*psb*C light-responsive promoter. Lower fluences of blue light (Fig. 2), given for extended periods of time (about 8 h), and high-fluence red light at >200 μmol m^{-2} s^{-1} did not induce the promoter (our unpublished results). The requirement for high-fluence blue light signifies that additional photosensory factors, unique from low-fluence components, are involved in light-intensity perception.

It is conceivable that the activation of the *psb*D-*psb*C light-responsive promoter by blue light could occur though a positive feedback control mechanism involving photosynthesis. However, the ability of blue light to activate *psb*D-*psb*C expression was not altered in the Chl *a*-minus mutant, xan-f^{10}, which lacks photosynthetic activity (Gamble and Mullet, 1989b). Moreover, the red-light treatments used here stimulate Chl biosynthesis, chloroplast development, and photosynthesis (data not shown) and also, in pea, *RbcS* and *Lhca/b* gene expression (Kaufman, 1993). However, these red-light treatments did not induce the *psb*D-*psb*C light-responsive promoter. Chloramphenicol pretreatments, which prevented the development of photosynthetically competent chloroplasts, did not alter the blue-light response (Gamble and Mullet, 1989b). Hence, the regulation of *psb*D-*psb*C expression by blue light is not dependent on photosynthetic electron flow. Instead, based on the ability of cycloheximide to inhibit blue-light induction of *psb*D-*psb*C (Gamble and Mullet, 1989b), blue light is proposed to regulate the expression of nuclear gene(s) encoding chloroplast factors that control the light-responsive promoter.

Selective Light-Mediated Turnover of D2, CP43, and D1 in Barley Chloroplasts

We present evidence for the selective light-mediated turnover of PSII subunits D2, CP43, and D1 in barley chloroplasts. In terms of photobiology, protein turnover had different wavelength and fluence requirements than did activation of *psb*D-*psb*C transcription. Light fluences of 200 to 350 μmol m^{-2} s^{-1} were required regardless of spectra tested. We estimated that full sunlight of 2100 μmol m^{-2} s^{-1} provides

approximately 240 μmol m^{-2} s^{-1} of blue and 280 μmol m^{-2} s^{-1} of red light. Therefore, the high-fluence white, blue, and red light treatments used here (220–350 μmol m^{-2} s^{-1}), which caused turnover of D1, D2, and CP43, were similar to fluences that plants receive in nature. In *Spirodela* (Greenberg et al., 1989; Mattoo et al., 1989), fluence-dependent D1 turnover occurs at much lower fluences (30 μmol m^{-2} s^{-1}) than were effective in barley. This difference could be due to differences in leaf and frond orientation to the light source and species-specific photoregulatory and photoprotective processes (Anderson, 1986; Demming-Adams and Adams, 1992).

Significant degradation occurred under equal fluences of red or blue light. However, the kinetics and degree of degradation was enhanced by blue light for D1, D2, and CP43. Red light was less effective, especially during the first 20 h of treatment. Degradation under blue light (220 μmol m^{-2} s^{-1}) and UV-A (380 μmol m^{-2} s^{-1}) closely resembled degradation under white light (350 μmol m^{-2} s^{-1}). We estimated that the white light source (350 μmol m^{-2} s^{-1}) provided about 40 μmol m^{-2} s^{-1} of blue light. When this lower fluence rate of blue light was applied, very little degradation was observed. Hence, this may suggest an additive or synergistic effect of blue light when combined with other components of white light.

Light-induced D2 degradation has been reported in higher plants (Mattoo et al., 1989) and algae (Schuster et al., 1988) in vivo. D2 was reported to be 10-fold more stable than D1, with turnover rates increasing markedly with increasing light intensity (Mattoo et al., 1989). We observed here that in plants exposed to red, blue, or white light, D1 and D2 were degraded at similar rates. In barley seedlings exposed to 350 μmol m^{-2} s^{-1} white light, the relative half-lives of D2 and D1 were each estimated to be 9.5 to 10 h. These values most closely resemble the half-life of the granal form of D1, which is 6 to 12 h in *Spirodela* grown at 30 μmol m^{-2} s^{-1} (Mattoo et al., 1989). Much higher rates of D1 turnover have been reported in nascent stromal fractions and under different irradiation regimes (Greenberg et al., 1989).

The rates of D1 and D2 degradation were similar under visible light but not under UV-A radiation. D1 was more labile in UV-A than in D2. The differential sensitivity of these closely associated subunits to UV-A and the differential effects of red and blue light may be evidence for multiple photosensitive targets. There is precedent for separate photosensitizers mediating PSII subunit decay. The in vivo degradation of D1 is enhanced under UV radiation, especially in white light containing UV-B (Greenberg et al., 1989). Two photosensitizers, Chl (donor-side inhibition) (Greenberg et al., 1989; Barber and Andersson, 1992) and quinone (acceptor-side inhibition) (Greenberg et al., 1989; Melis et al., 1992), have been implicated in light-induced damage of D1. Chl may mediate the effects of red light and quinone may mediate the effects of UV. Greenberg et al. (1989) have proposed that UV radiation may preferentially alter the redox state of quinones associated with D1, leading to damage and degradation of this protein. Additional evidence from experiments with isolated spinach thylakoids (Melis et al., 1992) supports the view that plastoquinone is a target of UV-B-induced damage to PSII. The enhanced degradation of D2 (and D1) under blue light observed here may also occur by way of quinone-activated destabilization. In this regard it is interesting to note that the rate and extent of CP43 degradation was less than that of D2 and D1, which may indicate a difference in the susceptibility of CP43, which lacks quinone, to the damaging effects of light.

The turnover of CP43 may be a consequence of D2 and D1 turnover. Furthermore, turnover of D2 and CP43 may be influenced by D1 turnover. By inhibiting D1 synthesis with chloramphenicol, replacement of degraded D1 was prevented. D2 and CP43 may then have been destabilized as a consequence of D1 vacancy, as observed in D1-deficient mutants (Vermaas and Ikeuchi, 1991). As part of normal PSII repair, the close association of D2 with D1 may predispose D2 to being replaced and reassembled with D1, Chl, quinones, and other cofactors and apoproteins. Hence, adequate levels of D2 would be needed to reassociate with newly synthesized D1. Under UV-A, the decay of D2 and CP43 is slower than and separable from D1 decay (Fig. 6). Initially, different photosensitizer molecules (Chl, Q_A, Q_B) may independently trigger degradation of D2 and D1. The effects of depletion of D1 from the PSII complex is expected to decrease the stability of the remaining subunits. A more general destruction of PSII would then result. In this way, extremes in the effects of blue and red light may become diminished by 25 h (Fig. 6). The mutually influential effects would be especially damaging under photoinhibitory light or environmental stresses (Feierabend et al., 1992) that inhibit chloroplast protein synthesis.

Role of a Blue-Light/UV-A-Regulated Promoter in Chloroplasts

The *psb*D-*psb*C operon consists of multiple overlapping transcription units that are required at different stages of chloroplast development. Three *psb*D-*psb*C promoters are active in dark-grown and illuminated seedlings (Sexton et al., 1990). They produce mRNAs that are utilized for the buildup and assembly of functional PSII during thylakoid membrane formation. Once PSII is assembled and mature chloroplasts are formed, the activity of these three promoters and the levels of corresponding mRNAs gradually decrease. A fourth promoter, the blue-light-responsive promoter (Christopher et al., 1992), is differentially activated by blue light and UV-A and assumes the dominant role of *psb*D-*psb*C transcription in mature chloroplasts.

As chloroplasts mature, the level of *psb*D-*psb*C mRNA is maintained by blue-light/UV-A-activated transcription, whereas *psb*A mRNA is maintained at high levels due to light-independent RNA stabilization (Rapp et al., 1992; Kim et al., 1993). In mature chloroplasts, the mRNAs that arise from the blue-light-responsive promoter constitute over 90% of the total *psb*D-*psb*C mRNA population (Fig. 1, lane 4; Christopher et al., 1992). Based on polysomal mRNA analysis (Mullet et al., 1990), more than 90% of the blue-light-induced *psb*D-*psb*C mRNAs are recruited onto polysomes. Therefore, the blue-light-induced *psb*D-*psb*C mRNAs provide the major mRNA coding capacity for D2 and CP43 in mature chloroplasts. This is significant because the light-mediated degra-

dation of D1, D2, and CP43 is correlated with the differential maintenance of their mRNA levels, the association of these mRNAs with polysomes (Klein et al., 1988; Mullet et al., 1990), and sustained translation rates (Zhu et al., 1984; Mullet et al., 1990; Droillard et al., 1992). Moreover, the levels of D1, D2, and CP43 are maintained in thylakoids of high-light-grown seedlings not treated with chloramphenicol (Figs. 3 and 4), an indication that the subunits are continuously replaced despite their instability in the light. Taken together, these data support the conclusion that the blue-light-responsive promoter functions to maintain *psb*D-*psb*C mRNA levels in mature chloroplasts to meet the demand for production of D2 and CP43 in illuminated plants. This conclusion is further supported by genetic experiments (Bustos and Golden, 1992) in which a light-regulated *psb*D gene was shown to be important for survival of cyanobacteria under high light. We suggest that in plants a high-fluence blue-light signaling pathway plays a critical role in maintaining PSII function, especially under high-intensity light. Additional regulation could also occur at the translational level. In both cases, the increase in D2 and CP43 synthesis would allow plants to replace damaged PSII subunits.

It is tempting to speculate that a blue-light pathway is used to regulate *psb*D-*psb*C gene expression because gene product degradation is enhanced under blue light. However, we emphasize that gene activation and gene product degradation are not part of the same photosensory pathway and that D1, D2, and CP43 degradation do not appear to activate the promoter. Most likely, transcription and protein turnover are co-regulated by light and have co-evolved as part of a mechanism to sustain PSII function. This regulation may also allow plants to alter PSII subunit stoichiometry in response to varying light conditions (Anderson, 1986). Such photoregulated adaptation could also be important for adjusting the ratio of core subunits to antennae, maximizing photosynthetic yields.

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