The EMBO Journal vol.8 no.10 pp.2785-2794, 1989

Blue light regulates the accumulation of two *psbD-psbC* transcripts in barley chloroplasts

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Communicated by J.Weil

Synthesis of D2, a Photosystem II reaction center protein encoded by *psbD*, is differentially maintained during light-induced chloroplast maturation. The continued synthesis of D2 is paralleled by selective light-induced accumulation of two *psbD-psbC* transcripts which share a common 5′ terminus. In the present study, we examine the nature of the photoreceptor and the fluence requirement for *psbD-psbC* transcript induction. The light-induced change in *psbD-psbC* RNA population can be detected between 1 and 2 h after 4.5 day old dark-grown barley seedlings are transferred to the light. Light-induced transcript accumulation occurs normally in the chlorophyll-deficient barley mutant, xan-f^{10}, indicating that light-activated chlorophyll formation and photosynthesis are not required for RNA induction. High fluence blue light fully induces *psbD-psbC* transcript accumulation; low or high fluence red or far-red light do not. However, *psbD-psbC* transcript accumulation elicited by blue light pulses can be partially attenuated if far-red light is given immediately following the blue light treatment. Thus, although blue light is needed to initiate transcript accumulation, phytochrome modulates the amplitude of the response. Pretreatment of dark-grown plants with cycloheximide blocks light-induced *psbD-psbC* transcript accumulation. This could implicate a blue-light responsive nuclear gene in the light-induced accumulation of the two *psbD-psbC* transcripts.

Key words: barley/blue light/chloroplast/RNA

Introduction

Higher plant development is regulated by at least three photoreceptors, phytochrome, the protochlorophyllide holochrome, and cryptochrome (for reviews, see Senger, 1982; Tobin and Silverthorne, 1985; Harpster and Apel, 1985; Ellis, 1986; Thompson *et al.*, 1986). Phytochrome is a chromoprotein consisting of a linear tetrapyrrole chromophore covalently linked to a polypeptide of 120-127 kd depending on the plant species (Rudiger and Scheer, 1983; Vierstra *et al.*, 1984). It can exist in either of two photointerconvertible forms: a red absorbing form (P_r), with an absorption maximum near 667 nm, and a far-red absorbing form (P_{fr}), with an absorption maximum near 730 nm. Photoconversion of P_r to P_{fr} by red light induces a diverse array of physiological responses, whereas reconversion of P_{fr} to P_r by far-red light will usually cancel the inductive effect (Hendricks and Van Der Woude, 1983). Examples of phytochrome-mediated photoresponses in plants include stem elongation, germination and phototropism (Ellis, 1986; Thompson *et al.*, 1986). In addition, phytochrome has been shown to regulate the expression of a number of nuclear genes in higher plants including those encoding the chlorophyll *a/b*-binding proteins of the light-harvesting complex (Thompson *et al.*, 1983; Horwitz *et al.*, 1988; Mosinger *et al.*, 1988), the small subunit of ribulose bisphosphate carboxylase (Thompson *et al.*, 1983), protochlorophyllide reductase (Apel, 1981; Mosinger *et al.*, 1988) and phytochrome (Colbert *et al.*, 1985). The expression of a number of plastid-encoded genes has been shown to be regulated by phytochrome as well (Link, 1982; Tobin and Silverthorne, 1985; Zhu *et al.*, 1985).

A second red light photoreceptor in higher plants is the protochlorophyllide holochrome. Protochlorophyllide and NADPH bind to protochlorophyllide reductase. This ternary complex has an absolute requirement for light before it becomes catalytically active (Griffiths and Oliver, 1984). Upon exposure to red light, protochlorophyllide is reduced to chlorophyllide and subsequently esterified to yield chlorophyll *a* (Castelfranco and Beale, 1983). Illumination of etiolated plants causes the photoreduction of protochlorophyllide, chlorophyll *a* accumulation and the conversion of the crystalline configuration of the prolamellar body into primary lamellar membranes (Henningsen and Stummann, 1982). Furthermore, the protochlorophyllide holochrome is involved in the control of chlorophyll biosynthesis (Harpster and Apel, 1985) and in the accumulation of the chlorophyll *a* apoproteins (Klein *et al.*, 1988).

The photoreceptor, cryptochrome, is specific for the UV/blue light region of the spectrum. The nature of cryptochrome is unknown, although flavin derivatives have been suggested as the possible photoreceptor (Briggs and Iino, 1983). Unlike the two red light photoreceptors which are predominantly found in higher plants, blue light photoreceptors are widely distributed and are found in fungi, yeast, halobacteria, mammals and plants (for review see Senger, 1984). Excitation of the blue light receptor is known to control processes such as vegetative reproduction (Corrochano *et al.*, 1988) and phototropism (Galland and Lipson, 1987; Kubo and Mihara, 1988) in the fungus *Phycomyces*, chloroplast movement in the alga *Vaucheria* (Blatt, 1983), photosynthetic activity in *Acetabularia* (Wennicke and Schmid, 1987), and carotenogenesis in *Phycomyces* and *Neurospora* (Lopez-Diaz and Cerda-Olmedo, 1980; Harding and Turner, 1981). In higher plants, cryptochrome controls a variety of developmental processes including phototropic curvature (Baskin, 1986; Baskin and Iino, 1987), stomatal opening (Ogawa, 1981; Karlsson *et al.*, 1983; Karlsson, 1986; Assmann, 1988), inhibition of stem elongation (Cosgrove, 1985; Shinkle and Jones, 1988) and chloroplast differentiation in cultured cells (Richter, 1984; Richter and Wessel, 1985; Richter *et al.*, 1987). In addition, it has recently been demonstrated that blue light affects the expression of the nuclear genes encoding the small subunit

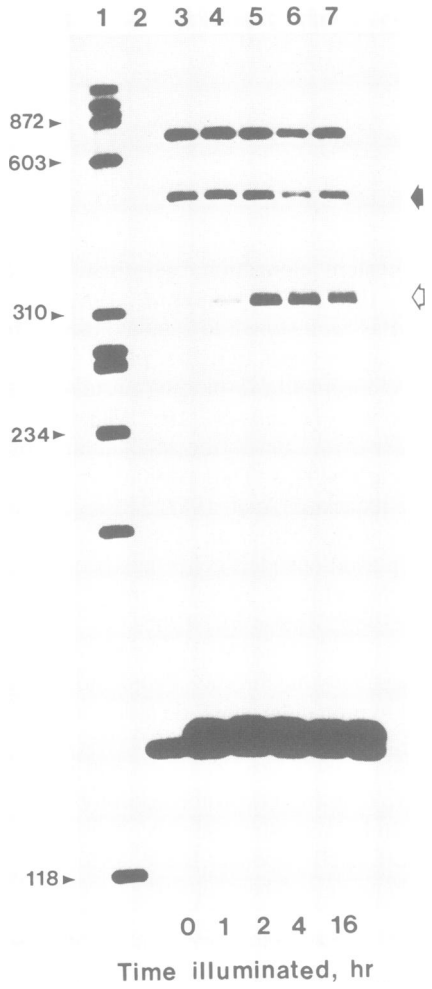

Fig. 1. Analysis of the 5′ terminus of the 4.0 and 3.2 kb *psbD-psbC* transcripts in plastids of dark-grown and illuminated barley seedlings. Numbers to the left of **lane 1** mark the sizes (bp) of some of the end-labeled ϕX174/*Hae*III fragments used as mol. wt markers. The 135 bp *Sau*96I−*Eco*RI DNA probe (5′ end-labeled at the *Eco*RI site) (**lane 2**) was hybridized to plastid RNA isolated from 4.5 day old dark-grown seedlings (**lane 3**) or seedlings transferred to an illuminated chamber (350 $\mu E/m^2/s$, fluorescent plus incandescent bulbs) for an additional 1 (**lane 4**), 2 (**lane 5**), 4 (**lane 6**) or 16 h (**lane 7**) and extended with AMV reverse transcriptase. The large, closed arrow to the right of lane 7 marks the 480 bp fragment corresponding to the 5′ terminus of the 4.1 and 3.3 kb *psbD-psbC* RNAs observed in plastids of dark-grown plants. The large, open arrow marks the 320 bp fragment corresponding to the 5′ end of the 4.0 and 3.2 kb *psbD-psbC* RNAs which accumulate upon illumination.

of ribulose bisphosphate carboxylase (Fluhr *et al.*, 1986; Fluhr and Chua, 1986) and the chlorophyll *a/b* binding proteins of the light-harvesting complex (Marrs and Kaufman, 1989).

The process of chloroplast biogenesis is regulated, in part, by the photoreceptors described above. In barley, primary leaf growth and accumulation of many of the polypeptides found in chloroplasts can occur in the absence of light (Klein and Mullet, 1986). For example, several of the polypeptides associated with PSII accumulate in plastids of dark-grown plants including those involved in oxygen evolution (OEE1, 2 and 3) and cytochrome b_{559} (Gamble and Mullet, 1989; Henningsen and Boardman, 1973). In contrast, synthesis and accumulation of other PSII polypeptides are regulated by light. Accumulation of the proteins encoded by the *psbA, psbB, psbC, psbD* and *cab* genes requires photoreduction of protochlorophyllide and subsequent chlorophyll *a* formation (Apel and Kloppstech, 1980; Bennett, 1981; Kreuz *et al.*, 1986; Klein *et al.*, 1988). Furthermore, *cab* gene transcription is regulated by phytochrome (Silverthorne and Tobin, 1984; Mosinger *et al.*, 1985; Steinmuller *et al.*, 1985). Thus, the two red light photoreceptors play a key role in coordinating synthesis and accumulation of the nuclear- and chloroplast-encoded polypeptides of PSII.

We recently reported that the PSII proteins encoded by the chloroplast *psbD* and *psbC* genes are co-transcribed from a complex transcription unit in barley (Berends *et al.*, 1987). In dark-grown plants, six RNAs are produced from this DNA region which hybridize to *psbD* and *psbC*. When dark-grown plants are illuminated, the level of the six *psbD-psbC* transcripts declines and two different RNAs accumulate (Gamble *et al.*, 1988). Furthermore, S1 nuclease and primer extension analyses indicated that the two light-induced *psbD-psbC* transcripts share a common 5′ end (Gamble *et al.*, 1988). The accumulation of the light-induced *psbD-psbC* transcripts in mature chloroplasts is correlated with the maintenance of high rates of *psbD* gene product synthesis in these plastids. It has been proposed that the maintenance of *psbD* gene product synthesis in mature chloroplasts is needed to replace subunits turned over as a consequence of PSII photochemistry (Gamble *et al.*, 1988).

In the present study, we examined the nature of the photoreceptor controlling the accumulation of the two *psbD-psbC* transcripts in barley chloroplasts. The results show that blue light (high fluence range) induces the accumulation of the two *psbD-psbC* RNAs. Accumulation of these transcripts was not significantly induced by red light. However, the blue light induction was partially reversed by far-red illumination. Finally, we show that cytoplasmic translation is required for the blue light induction of the two *psbD-psbC* transcripts suggesting that blue light may act by altering nuclear gene expression.

Results

Accumulation of the 4.0 and 3.2 kb psbD-psbC transcripts during light-induced chloroplast development

In a previous study, we characterized the transcripts which hybridize to the chloroplast *psbD* and *psbC* genes in plastids of dark-grown and illuminated barley seedlings (Gamble *et al.*, 1988). We found that illumination of 4.5 day old dark-grown seedlings resulted in a decline in the level of the six *psbD-psbC* transcripts found in etioplasts and the accumulation of two different transcripts of 4.0 and 3.2 kb which hybridize to *psbD* and *psbC*. When seedlings were illuminated with white light (350 $\mu E/m^2/s$) for <1 h, the two *psbD-psbC* transcripts were barely detectable. However, after 16 h of illumination marked accumulation of these transcripts was observed (Gamble *et al.*, 1988). In the present study, we have refined our earlier analysis of the time course for induction of the two *psbD-psbC* transcripts following illumination of dark-grown seedlings.

The 4.0 and 3.2 kb light-induced *psbD-psbC* transcripts have a common 5′ end ~100−150 nt downstream from the 5′ end of two *psbD-psbC* transcripts (4.1 and 3.3 kb in length) which accumulate in etioplasts (Gamble *et al.*, 1988). Due to the similarity in size between the 4.1 and 4.0 kb RNAs and the 3.3 and 3.2 kb RNAs it is difficult to

Fig. 2. Analysis of the 5′ terminus of the 4.0 and 3.2 kb *psbD-psbC* transcripts in plastids of wild-type and xan-f^{10} mutant seedlings. **Lane 1** shows the end-labeled ϕX174/*Hae*III fragments used as mol.wt markers. The 135 bp *Sau*96I−*Eco*RI DNA probe shown in **lane 2** was used for primer extension on plastid RNA isolated from 6 day old dark-grown wild type (**lane 3**) and mutant (**lane 5**) seedlings or dark-grown wild-type (**lane 4**) and mutant (**lane 6**) seedlings transferred to an illuminated chamber (350 μE/m^2/s, fluorescent plus incandescent bulbs) for an additional 4 h. Figure legends same as Figure 1.

distinguish these RNAs on agarose gels. Therefore, primer extension analysis was used to examine the accumulation of the 5′ terminus corresponding to the 4.0 and 3.2 kb RNAs during light-induced plastid development (Figure 1). A 135 bp *Sau*96I−*Eco*RI DNA fragment (5′ end-labeled at the *Eco*RI site) (lane 2) was used to probe plastid RNA from dark-grown seedlings (lane 3) and dark-grown seedlings illuminated with white light (350 μE/m^2/s) for 1, 2, 4 or 16 h (lanes 4−7). When plastid RNA from dark-grown seedlings was used for the primer extension assay, two predominant protected fragments of ~800 and 480 bp as well as a faint band of ~320 bp were generated (lane 3). The origin of the 800 bp fragment is unknown; however, the level of this fragment was not altered following illumination (c.f. lane 3 and lanes 4−7). The 480 bp fragment (marked by closed arrow) corresponds to the 5′ terminus of the 4.1 and 3.3 kb RNAs which accumulate in plastids of dark-grown seedlings (lane 3). The 320 bp fragment (marked by open arrow) marks the 5′ terminus of the light-induced 4.0 and 3.2 kb *psbD-psbC* transcripts (lane 3). We have consistently observed very low levels of these two transcripts in all etioplast populations examined (Gamble *et al.*, 1988). When dark-grown seedlings were illuminated with white light for 1 h, a slight increase in the level of the 320 bp fragment was observed (lane 4). However, after 2 h of continuous illumination, the level of the 320 bp band increased markedly (lane 5). These results indicate that light-induced accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts requires 1−2 h of continuous illumination with white light. In some experiments a protected fragment of 350 bp was observed (Figures 3−6). The origin of this protected fragment is unclear. In addition, we do not know why this band is observed in some experiments but not others.

Accumulation of the two psbD-psbC transcripts in the chlorophyll-deficient mutant, xan-f^{10}

Recently, Klein *et al.* (1988) demonstrated that accumulation of the protein encoded by *psbC* requires chlorophyll *a* biosynthesis, and that the protochlorophyllide holochrome is one red light photoreceptor controlling this response. To investigate whether the light-induced accumulation of the two *psbD-psbC* transcripts also requires chlorophyll *a* or chlorophyll precursors, their accumulation in the chlorophyll-deficient barley mutant, xan-f^{10}, was examined. Mutants of the xan-f loci are blocked in the biosynthesis of chlorophyll *a* at the conversion of protoporphyrin IX to Mg-protoporphyrin IX (von Wettstein *et al.*, 1971; Henningsen and Stummann, 1982). Mutant, xan-f^{10} fails to accumulate protochlorophyllide in the dark and is devoid of chlorophyll *a* in the light (Klein *et al.*, 1988). In Figure 2 the accumulation of the 5′ end corresponding to the 4.0 and 3.2 kb *psbD-psbC* transcripts in plastids from dark-grown and illuminated xan-f^{10} mutant seedlings is shown. Plastids from dark-grown wild-type and mutant seedlings contain very low amounts of the 320 bp fragment (lanes 3 and 5, marked by open arrow). However, continuous illumination of either wild-type or xan-f^{10} mutant seedlings for 4 h with white light (350 μE/m^2/s) results in a significant increase in the level of the 320 bp fragment (lanes 4 and 6) indicating that light-induced accumulation of the two *psbD-psbC* transcripts does not require chlorophyll *a* or chlorophyll precursors (i.e. Mg-protoporphyrin IX, protochlorophyllide or chlorophyllide). Furthermore, these results indicate that accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts during chloroplast development is not controlled by the protochlorophyllide holochrome.

Phytochrome and cryptochrome control of psbD-psbC transcript levels

We next checked the involvement of phytochrome and cryptochrome in the light-induced accumulation of the two *psbD-psbC* transcripts. To determine whether phytochrome mediates the increase in the level of the 4.0 and 3.2 kb RNAs, dark-grown seedings were irradiated with a pulse of red light and the accumulation of the 5′ end corresponding to the 4.0 and 3.2 kb RNAs examined. As shown in Figure 3A, a 30 s pulse of red light (500 μE/m^2) followed by 4 h of darkness resulted in a very slight increase in the level of the 320 bp protected fragment compared to dark-grown seedlings (cf. lanes 3 and 4, marked by open arrow). The induction by red light was reversed by far-red light (1200 μE/m^2, 1 min duration) given immediately following the red light pulse (lane 5). Finally, a pulse of far-red light

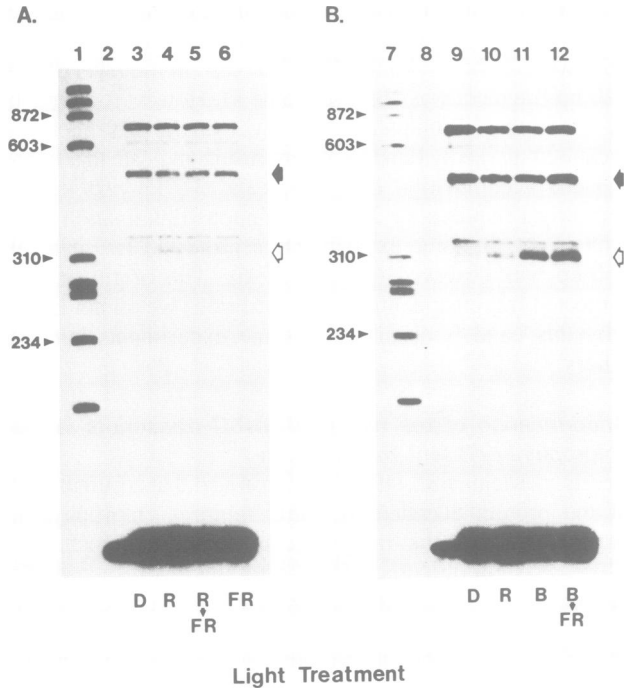

Fig. 3. Effect of light quality on the accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts in barley seedlings. Numbers to the left of **lanes 1** and **7** indicate the sizes (bp) of some of the end-labeled ϕX174/*Hae*III fragments used as mol. wt markers. The 135 bp *Sau*96I−*Eco*RI DNA probe (**lanes 2** and **8**) was used for the primer extension assays shown in lanes 3−6 and 9−12. (**A**) 4.5 day old dark-grown seedlings were illuminated with red light (500 μE/m^2 over 30 s period). Following irradiation with red light, plants were either returned to darkness for 4 h or were irradiated immediately with far-red light (1200 μE/m^2 over 1 min period). Red plus far-red treated plants were then placed in darkness for 4 h. A third set of seedlings was irradiated with far-red light alone (1200 μE/m^2 over 1 min) and then returned to darkness for 4 h. Subsequently, RNA was isolated from leaf tissue as described in Materials and methods. Each lane contained 10 μg of leaf RNA. Irradiation treatments: dark, **lane 3**; red light, **lane 4**; red plus far-red light, **lane 5**; far-red light alone, **lane 6**. (**B**) 4.5 day old dark-grown seedlings were irradiated continuously for 2 h with red light (2.12 × 10^5 μE/m^2) or blue light (2.12 × 10^5 μE/m^2). In one experiment, the continuous blue light was supplemented with 5 min of far-red illumination (6000 μE/m^2) every 20 min. Intact plastids were isolated at the end of the light treatments and plastid nucleic acid extracted. Irradiation treatments, dark, **lane 9**; 2 h red light, **lane 10**; 2 h blue light, **lane 11**; 2 h blue light supplemented with short flashes of far-red light, **lane 12**. Figure legends same as Figure 1.

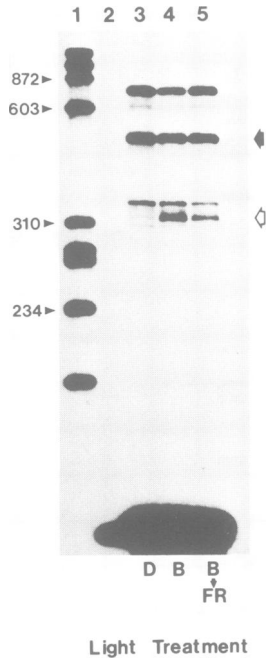

Fig. 4. Effect of far-red light on the blue light-mediated accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts. 4.5 day old dark-grown barley seedlings were irradiated with a pulse of blue light (1.5 × 10^3 μE/m^2 over 5 min) every 20 min for 4 h. Another set of seedlings was irradiated with a pulse of blue light (1.5 × 10^3 μE/m^2 over 5 min) followed immediately by a pulse of far-red light (7200 μE/m^2 over 5 min) every 20 min for 4 h. Following the irradiation treatments, intact plastids were isolated and plastid nucleic acid extracted. The end-labeled ϕX174/*Hae*III fragments used as mol. wt markers are shown in **lane 1. Lane 2** shows the 135 bp *Sau*96I−*Eco*RI DNA probe used for the primer extension assays in lanes 3−5. Irradiation treatments: dark, **lane 3**; blue light, **lane 4**; blue plus far-red light, **lane 5**. Figure legends same as Figure 1.

alone (1200 μE/m^2) followed by 4 h of darkness was not effective in inducing the accumulation of the 4.0 and 3.2 kb RNAs (lane 6). It has previously been demonstrated that a pulse of red light (500 μE/m^2) results in an 18-fold increase in *cab* mRNA levels compared to levels in dark-grown seedlings, and that far-red light administered immediately after the red light pulse partially attenuates this response (Klein *et al.*, 1988). Thus, while the fluence of red light given in this experiment is able to induce the low fluence phytochrome component of *cab* mRNA accumulation, it is much less effective in inducing the accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts.

It has been well established that a number of phytochrome- and blue light-mediated processes are high irradiance reaction responses which require prolonged irradiation with red or blue light respectively (Thomas and Dickinson, 1979; Holmes and Schafer, 1981; Cosgrove, 1982; Heim and Schafer, 1982; Holmes *et al.*, 1982; Mancinelli, 1985). Therefore, the effect of continuous red or blue irradiation on the accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts was next examined. For this experiment, dark-grown seedlings were illuminated continuously for 2 h with an equal fluence (2.12 × 10^5 μE/m^2) of red or blue light. Following irradiation, plastids were isolated and plastid RNA assayed by primer extension analysis (Figure 3B). When dark-grown seedlings were illuminated for 2 h with red light, a slight increase in the level of the 320 bp fragment was observed compared to the level in plastids from dark-grown seedlings (cf. lanes 9 and 10, marked by open arrow). In comparison, illumination of seedlings for 2 h with blue light resulted in marked accumulation of the 320 bp protected fragment (lane 11) indicating that accumulation of the two *psbD-psbC* transcripts is mediated by blue light. Since both the P_r and P_{fr} forms of phytochrome are capable of absorbing blue light (Schafer and Haupt, 1983), the effect of blue light on the accumulation of the two *psbD-psbC* transcripts could also involve phytochrome. To test this possibility, we examined the ability of far-red light to reverse the blue light-mediated increase in the level of the 4.0 and 3.2 kb RNAs. Supplementation of continuous blue light with short pulses of far-red light (6000 μE/m^2 over 5 min) every 20 min did not attenuate the accumulation of the two transcripts (cf. lanes 11 and 12).

Effect of far-red irradiation on the blue light-mediated accumulation of the two psbD-psbC transcripts

The primer extension assays presented in Figure 3 show that the blue light-mediated increase in the level of the two *psbD-psbC* transcripts was not reversed by far-red irradiation suggesting that the predominant photoreceptor controlling this process is cryptochrome. In that experiment, seedlings were irradiated continuously for 2 h with blue light (2.12×10^5 $\mu E/m^2$) which was supplemented every 20 min with short flashes of far-red light. However, if the escape time from P_{fr} control is <20 min, then the experiment shown in Figure 3 would not reveal the involvement of phytochrome in the accumulation of the two *psbD-psbC* transcripts. Therefore, dark-grown seedlings were irradiated with either a pulse of blue light (1.5×10^3 $\mu E/m^2$ over 5 min) or a pulse of blue light (1.5×10^3 $\mu E/m^2$) followed by a pulse of far-red light (7200 $\mu E/m^2$ over 5 min) every 20 min over a 4 h period. Following the irradiation treatments, plastid RNA was isolated and analyzed by primer extension analysis (Figure 4). Illumination of dark-grown seedlings with pulses of blue light (1.5×10^3 $\mu E/m^2$) every 20 min for 4 h resulted in an increase in the level of the two *psbD-psbC* transcripts over levels in the dark (cf. lanes 3 and 4). Far-red light (7200 $\mu E/m^2$) administered immediately following the blue light pulse caused a partial attenuation in the accumulation of the two transcripts (cf. lanes 4 and 5). Therefore, while accumulation of the two *psbD-psbC* transcripts is controlled predominantly by a blue light photoreceptor, this process is influenced to some extent by phytochrome. Furthermore, these results indicate that the escape from P_{fr} control is <20 min (cf. Figure 3, lane 12 and Figure 4, lane 5).

Fluence response relationship of psbD-psbC transcript accumulation

Figure 5 shows the fluence response relationship for the accumulation of the two *psbD-psbC* transcripts induced by blue light. Dark-grown seedlings were irradiated continuously for 2 h with various fluences of blue light and the accumulation of the two transcripts examined. In this experiment, total fluence was varied by changing the fluence rates of blue light with the aid of neutral density filters. As shown in Figure 5, the 4.0 and 3.2 kb RNAs were undetectable in dark-grown seedlings (lane 3, marked by open arrow). A protected fragment of ~350 bp was observed in all of the samples analyzed (lanes 3–8, marked by small arrowhead). The level of this fragment is not modulated by blue light (Figure 5, cf. lane 3 and lanes 4–8). The threshold for accumulation of the two *psbD-psbC* transcripts was ~1.85×10^3 $\mu E/m^2$ blue light and was saturated between 1.06×10^4 and 1.06×10^5 $\mu E/m^2$ (lanes 5 and 6). Although not shown in Figure 5, fluences <1.85×10^3 $\mu E/m^2$ were not effective in inducing the response (data not shown). The results also show that 2 h of continuous blue light (1.06×10^5–2.12×10^5 $\mu E/m^2$) was just as effective in eliciting an increase in the level of the two *psbD-psbC* RNAs as 2 h of continuous white light (2.34×10^6 $\mu E/m^2$) (cf. lanes 6 and 7 with 8). The high fluence of blue light necessary for accumulation of the two *psbD-psbC* transcripts is characteristic of a high irradiance reaction response (Mancinelli, 1985).

Fig. 5. Blue light fluence response of *psbD-psbC* transcript accumulation. 4.5 day old dark-grown barley seedlings were illuminated with different fluences of blue light continuously for 2 h or with white light for 2 h. Following irradiation, intact plastids were isolated and plastid nucleic acid extracted. **Lane 1** shows the end-labeled ϕX174/*Hae*III fragments used as mol. wt markers. The 135 bp *Sau*96I–*Eco*RI DNA probe (**lane 2**) was used for the primer extension assays shown in lanes 3–8. Irradiation treatments: dark, **lane 3**; 1.85×10^3 $\mu E/m^2$ blue light, **lane 4**; 1.06×10^4 $\mu E/m^2$ blue light, **lane 5**; 1.06×10^5 $\mu E/m^2$ blue light, **lane 6**; 2.12×10^5 $\mu E/m^2$ blue light, **lane 7**; 2.34×10^6 $\mu E/m^2$ white light, **lane 8**. Figure legends same as Figure 1 plus small dark arrowhead marks the position of a 350 bp protected fragment of unknown origin.

Accumulation of the two psbD-psbC transcripts in AFPA-treated seedlings

Recently, Gardner *et al.* (1988) demonstrated that the transaminase inhibitor, 4-amino-5-fluoropentanoic acid (AFPA) inhibits phytochrome synthesis in dark-grown pea, corn and oat seedlings. In the present study, this inhibitor was used to further test the involvement of phytochrome in the accumulation of the two *psbD-psbC* transcripts. Dark-grown seedlings were grown in the presence of 1 mM AFPA to inhibit phytochrome synthesis and then illuminated with white light (350 $\mu E/m^2/s$) for 4 h. Subsequently, total leaf nucleic acid was isolated and analyzed for the presence of the two light-induced *psbD-psbC* RNAs. As shown in Figure 6, AFPA treatment of seedlings did not inhibit accumulation of the two *psbD-psbC* transcripts (cf. lanes 5 and 6). It has recently been demonstrated that treatment of barley seedlings with 1 mM AFPA inhibits 80% of the low fluence phytochrome component of *cab* mRNA accumulation (Rapp and Mullet, personal communication). Therefore, these results indicate that either the accumulation of the two *psbD-psbC* RNAs is not significantly influenced by phytochrome in continuously illuminated plants or that the level of phytochrome synthesized in AFPA-treated seedlings

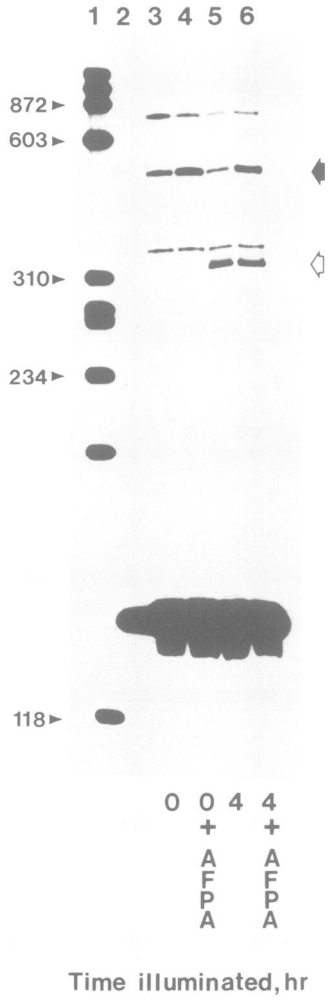

Fig. 6. Effect of AFPA treatment on the accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts. Barley seeds were germinated in complete darkness on paper towels saturated with Hoagland's nutrient solution. After 2 days, seedlings were transplanted to beakers containing paper soaked with either water or 1 mM AFPA. Growth was continued for another 3 days in complete darkness and then seedlings were transferred to an illuminated chamber (350 $\mu E/m^2/s$) for an additional 0 or 4 h. Following the irradiation treatments, total leaf nucleic acid was extracted as described in Materials and methods. Each lane contained 10 μg of leaf RNA. The end-labeled ϕX174/*Hae*III fragments used as mol. wt markers are shown in **lane 1**. The 135 pb *Sau*96I–*Eco*RI DNA probe (**lane 2**) was used for the primer extension assays shown in lanes 3–6. Irradiation and inhibitor treatments: dark, **lane 3**; dark + AFPA, **lane 4**; 4 h white light, **lane 5**; 4 h white light + AFPA, **lane 6**. Figure legends same as Figure 1.

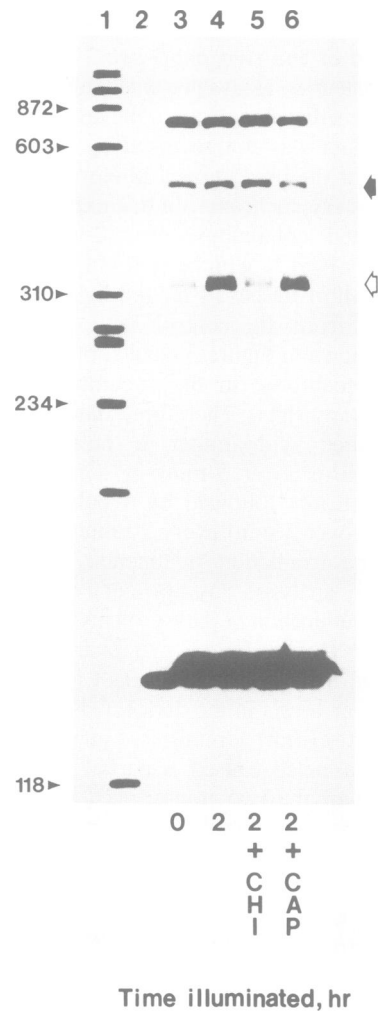

Fig. 7. Effect of cycloheximide and chloramphenicol on the accumulation of the light-induced *psbD-psbC* transcripts. Dark-grown barley leaves were excised and incubated in 40 mM HEPES–KOH with or without cycloheximide (20 $\mu g/ml$) or chloramphenicol (100 $\mu g/ml$) for 1 h prior to illumination with white light (350 $\mu E/m^2/s$). After illumination for 2 h, intact plastids were isolated and plastid nucleic acid extracted. **Lane 1** shows the end-labeled ϕX174/*Hae*III fragments used as mol. wt markers. The 135 bp *Sau*96I–*Eco*RI DNA probe (**lane 2**) was used for the primer extension assays shown in lanes 3–6. Irradiation and antibiotic treatments: dark, **lane 3**; 2 h white light, **lane 4**; 2 h white light plus cycloheximide, **lane 5**; 2 h white light plus chloramphenicol, **lane 6**. Figure legends same as Figure 1.

is sufficient to allow accumulation of these two transcripts upon illumination.

Effect of cycloheximide and chloramphenicol on the accumulation of the two psbD-psbC transcripts

Chloroplast biogenesis requires the synthesis of both nuclear- and chloroplast-encoded polypeptides. Each of the four photosynthetic complexes consists of subunits which are encoded on either chloroplast or nuclear genes (Gounaris *et al.*, 1986). In addition, a number of studies have shown that nuclear-encoded proteins are involved in the synthesis and assembly of functional photosynthetic complexes (for reviews, see Somerville, 1986; Rochaix and Erickson, 1988). To determine whether the light-induced accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts requires concomitant synthesis of either nuclear- or chloroplast-encoded proteins, we characterized the accumulation of these two RNAs from seedlings treated with the protein synthesis inhibitors, cycloheximide and chloramphenicol. For this experiment, dark-grown barley leaves were incubated with cycloheximide or chloramphenicol for 1 h prior to transfer to white light (350 $\mu E/m^2/s$, fluorescent plus incandescent bulbs). After 2 h in the light, plastids were isolated and plastid nucleic acid extracted and analyzed by primer extension assays. As shown in Figure 7, treatment of dark-grown seedlings with the chloroplast protein synthesis inhibitor, chloramphenicol, prior to illumination did not alter the light-induced increase in the level of the two *psbD-psbC* transcripts compared to control seedlings (cf. lanes 4 and 6). In contrast, treatment of seedlings with cycloheximide, an inhibitor of cytoplasmic protein synthesis, prevented accumulation of the two *psbD-psbC* RNAs (cf. lanes 4 and

5). These results suggest that the light-induced accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts requires concomitant synthesis of nuclear- but not chloroplast-encoded polypeptides.

Discussion

We have previously reported (Gamble *et al.*, 1988) that illumination of 4.5 day old dark-grown barley causes a change in the *psbD-psbC* transcript population. Dark-grown barley seedlings contain six RNAs which hybridize to the *psbD-psbC* transcription unit (Berends *et al.*, 1987; Gamble *et al.*, 1988). Upon illumination, the level of the six *psbD-psbC* transcripts observed in etioplasts declines and two different RNAs of 4.0 and 3.2 kb accumulate (Gamble *et al.*, 1988). To date, the light-induced accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts is the only example of a tightly light-regulated change in plastid transcript population observed in barley. Our previous results indicated that the two light-induced *psbD-psbC* transcripts have a common 5′ end (Gamble *et al.*, 1988). At present, it is not known whether the light-induced accumulation of this 5′ end results from RNA processing or a change in RNA transcription. These observations led us to determine the nature of the photoreceptor controlling the shift in the *psbD-psbC* transcript population.

In the present study, it was demonstrated that accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts was not significantly induced by red light. One photoreceptor known to absorb red light is phytochrome (Schwartzbach and Schiff, 1983; Hoober, 1984; Harpster and Apel, 1985). Phytochrome responses include both very low fluence (threshold $\sim 10^{-4}$ $\mu E/m^2$ red light) and low fluence (threshold of ~ 1 $\mu E/m^2$) components. We found that red light in the low fluence range was not effective in inducing the accumulation of the two *psbD-psbC* transcripts. This is unlike the light-induced accumulation of *cab* and small subunit mRNA both of which contain a low fluence phytochrome component (Kaufman *et al.*, 1986). In addition, high fluences of red light increased the level of the 4.0 and 3.2 kb *psbD-psbC* transcripts to only a small extent. We have also shown that the phytochrome synthesis inhibitor, AFPA (Gardner *et al.*, 1988), does not affect the light-induced accumulation of the two *psbD-psbC* transcripts, whereas *cab* mRNA accumulation is inhibited ~80% following AFPA treatment (Figure 6; Rapp and Mullet, personal communication). Although the amount of phytochrome synthesized in AFPA-treated seedlings may be sufficient to induce accumulation of the two *psbD-psbC* transcripts upon illumination, the fact that accumulation was induced to only a small extent by red light suggests that phytochrome is not the primary photoreceptor controlling the response.

A second red light absorbing photoreceptor in higher plants is the protochlorophyllide holochrome (Giffiths and Oliver, 1984; Harpster and Apel, 1985). Klein *et al.* (1988) have recently demonstrated that the protochlorophyllide holochrome is one red light photoreceptor controlling the accumulation of the chlorophyll apoproteins (proteins encoded by *psaA, psaB, psbA, psbB* and *psbC*) in barley seedlings. In the present study, it was found that accumulation of the two *psbD-psbC* transcripts was not governed by the protochlorophyllide holochrome. The barley mutant, xan-f^{10}, which is blocked in the biosynthesis of chlorophyll *a* prior to protochlorophyllide, accumulated wild-type levels of the two transcripts following illumination. Furthermore, since the xan-f^{10} mutant lacks photosynthetic activity (Henningsen and Stummann, 1982), these results indicate that accumulation of the 4.0 and 3.2 kb *psbD-psbC* RNAs is not coupled to photosynthetic function.

Accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts was induced by blue light. Fluence response studies showed that the threshold fluence of blue light was $\sim 1.85 \times 10^3$ $\mu E/m^2$ and that the response was saturated between 1×10^4 and 1×10^5 $\mu E/m^2$. The fluence of blue light required for the accumulation of the two *psbD-psbC* transcripts is characteristic of a high irradiance reaction response (HIR) (Mancinelli, 1985). A number of light-dependent processes in higher plants are induced by high-intensity blue light including anthocyanin production in seedlings (Mancinelli, 1985; Oelmuller and Mohr, 1985; Sponga *et al.*, 1986), inhibition of stem elongation in dicot seedlings (Meijer, 1968; Cosgrove, 1981; Gaba and Black, 1983), nitrite reductase activity in sorghum (Rajasekhar and Sopory, 1985) and second positive phototropism in oat (Everett and Thimann, 1968) and alfalfa (Baskin and Iino, 1987). Many HIR responses require prolonged exposures of light, but not necessarily continuous ones (Mancinelli, 1985). For example, Schafer *et al.* (1981) and Heim and Schafer (1982) have reported that for anthocyanin formation and hypocotyl elongation in mustard seedlings, continuous irradiations could be fully substituted by intermittent irradiations. Likewise, accumulation of the two *psbD-psbC* transcripts was induced either by continuous irradiation for 2 h with blue light or by intermittent pulses of blue light (5 min duration every 20 min) given over a 4 h period. In addition, the accumulation of the 4.0 and 3.2 kb *psbD-psbC* RNAs was similar to other HIR responses induced by continuous irradiation in that the response did not obey the Bunsen–Roscoe reciprocity law (data not shown).

A number of studies have indicated that the proteins encoded by *psbD* (D2) and *psbA* (D1) form a heterodimer which binds cofactors involved in primary charge separation (Deisenhofer *et al.*, 1984; Hearst and Sauer, 1984; Danielius *et al.*, 1987; Nanba and Satoh, 1987). In barley, the synthesis of D1, D2 and to a lesser extent, CP43 (*psbC* gene product) is differentially maintained in chloroplasts of plants illuminated for 72 to 108 h (Gamble *et al.*, 1988). Differential synthesis of these proteins is matched by *psbA* and *psbD-psbC* mRNA levels which remain elevated in these chloroplasts unlike most other plastid mRNAs (Klein and Mullet, 1987; Gamble *et al.*, 1988). Studies have shown that D1 (Mattoo *et al.*, 1984; Kyle *et al.*, 1984; Ohad *et al.*, 1985) and D2 (Schuster *et al.*, 1988) are less stable than other plastid proteins in illuminated chloroplasts. Thus continued synthesis of D1 and D2 in illuminated plants would be needed to maintain PSII activity. The turnover of D1 and D2 has not been examined in barley chloroplasts. We propose, however, that blue-light-induced synthesis of the 4.0 and 3.2 kb *psbD-psbC* transcripts allows continued synthesis of D2 which is probably required to replace subunits turned over in chloroplasts of illuminated barley plants.

The accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts requires activation of a blue light photoreceptor, since red and far-red irradiations were ineffective in inducing the response. However, these results do not exclude a possible role for phytochrome in the accumulation of the two

RNAs. In fact, experiments with simultaneous irradiations of blue and far-red light showed that accumulation of the two *psbD-psbC* transcripts was modulated by phytochrome. The blue light-induced increase in the level of the two RNAs was partially attenuated (~50%) when far-red light was given immediately following the blue light pulses. However, the escape from P_{fr} control was rapid since supplementation of 2 h continuous blue light with pulses of far-red light (5 min duration) every 20 min did not attenuate the blue light-induced increase in the level of the two *psbD-psbC* transcripts. Similarly, Kaufman *et al.* (1986) have observed that the accumulation of both *cab* RNA and pEA215 RNA begin to escape from P_{fr} control immediately following a red light pulse in developing pea buds. Thus, the present results demonstrate that two photoreceptors, cryptochrome and phytochrome, are responsible for the light-induced accumulation of the two *psbD-psbC* transcripts. Studies have shown that a number of light-dependent processes require the actions of both phytochrome and cryptochrome for full expression (for review, see Schafer and Haupt, 1983). Requirements for both cryptochrome and phytochrome have been noted for the induction of anthocyanin synthesis in tomato (Drumm-Herrel and Mohr, 1982; Sponga *et al.*, 1986) and milo seedlings (Oelmuller and Mohr, 1985), for hypocotyl growth in mustard (Cosgrove, 1982; Drumm-Herrel and Mohr, 1985), sesame (Drumm-Herrel and Mohr, 1984) and cucumber (Gaba *et al.*, 1984), and for the activation of β-amylase activity in mustard (Manga and Sharma, 1988) and nitrite reductase activity in sorghum (Rajasekhar and Sopory, 1985). In addition, maximal induction of *cab* (Marrs and Kaufman, 1989) and *rbcS* transcript levels (Fluhr and Chua, 1986; Fluhr *et al.*, 1986) requires the action of phytochrome as well as cryptochrome. In the above mentioned studies, it has been demonstrated that the light-mediated responses can be induced with either red or blue light. In contrast, only blue light initiates accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts in developing barley leaves; red light is ineffective in this respect. Similar observations have been reported in cultured plant cells where the accumulation of both nuclear- and chloroplast-encoded transcripts is induced solely by blue light (Richter, 1984; Richter and Wessel, 1985; Richter *et al.*, 1987).

Inhibition of cytoplasmic protein synthesis prevented the light-induced accumulation of the two *psbD-psbC* transcripts, whereas inhibition of chloroplast protein synthesis did not. This result suggests that the accumulation of the two *psbD-psbC* RNAs induced by blue light requires *de novo* synthesis of a nuclear-encoded gene product. Control could be at the level of nuclear-encoded gene transcription or translation. Alternatively, it is possible that cycloheximide is indirectly blocking the accumulation of the two *psbD-psbC* transcripts due to secondary effects of this inhibitor. However, analysis of nuclear photosynthetic mutants has revealed that nuclear gene products are involved in the regulation of chloroplast gene expression (for a recent review, see Rochaix and Erickson, 1988). For instance, Jensen *et al.* (1986) have characterized a nuclear mutant of *Chlamydomonas* that fails to accumulate *psbB* mRNA demonstrating that a nuclear gene product is involved in the accumulation of a specific chloroplast transcript. Similarly, Barkan *et al.* (1986) have shown that a nuclear mutant of maize, hcf-38, has a reduced amount and altered size distribution of *psbB* mRNA. In addition, this mutant exhibits reduced levels of *atpβ/ϵ*, *psaA* and *petA* RNA suggesting that the mutated nuclear gene product is involved in the transcription or processing of several chloroplast RNAs. Analogous fungal mutants have been described with nuclear lesions affecting the processing of specific mitochondrial transcripts (Dieckmann *et al.*, 1982; Faye and Simon, 1983; Garriga *et al.*, 1984). The present results suggest that a nuclear gene product may be involved in the expression of the *psbD-psbC* transcription unit. Experiments are currently underway to elucidate further the mechanism by which blue light induces the accumulation of the 4.0 and 3.2 kb *psbD-psbC* transcripts in barley chloroplasts.

Materials and methods

Plant growth and mutant isolation

Barley seeds (*Hordeum vulgare* L. var Morex) were planted in vermiculite and grown in a dark chamber located in a light-tight room as previously described (Gamble *et al.*, 1988). After 4.5 days in the dark, seedlings were either kept in darkness or were irradiated as described below.

Seeds of the barley mutant, xan-f[10], were planted in vermiculite and grown for 6 days in complete darkness. To identify mutant seedlings, the apical 0.5 cm of the primary leaf of dark-grown seedlings was excised under a dim, green safelight (Schiff, 1972) and transferred to an illuminated chamber (350 $\mu E/m^2/s$, fluorescent plus incandescent bulbs). After 12–16 h of illumination, the excised tips were visually scored for the presence of chlorophyll. After identifying mutant (chlorophyll-deficient, yellow) and wild-type seedlings, plants were either maintained in the dark or transferred to an illuminated chamber (350 $\mu E/m^2/s$, fluorescent plus incandescent bulbs), for 4 h prior to tissue harvest.

Light sources

The following light sources were used for experimental treatment.

(i) Red for pulse irradiation: slide projector light (Dukane 28A55 with 500 W Sylvania lamp; Dukane Corp., St Charles, IL) passed through a CBS Red filter (Carolina Biological Supply Co., Burlington, NC) which eliminates virtually all light below 600 nm.
(ii) Far-red for pulse irradiation: slide projector light passed through a CBS Far-red filter which eliminates virtually all light below 700 nm.
(iii) Blue for pulse irradiation: slide projector light passed through a blue glass filter (No. 5–60, 5 mm thick; Corning Glass Works, Corning, NY).
(iv) Red for continuous irradiation: light from white fluorescent lamps (F20T12/WW, Sylvania) passed through a layer of red Plexiglas (No. 2423, 3 mm thick; Rohm and Haas, Philadelphia, PA).
(v) Blue for continuous irradiation: light from blue fluorescent lamps (F20T12/Blue, Sylvania) passed through a layer of blue Plexiglas (No. 2424, 3 mm thick; Rohm and Haas).
(vi) White for continuous irradiation: light from fluorescent (F72T12/CW/HO, Sylvania) plus incandescent (Westinghouse 60 W) bulbs.

Photon fluence rates of red, blue and white light were measured using a quantum photometer (LI-190 SA; LI-COR Inc., Lincoln, NE). Energy fluences of far-red light were measured using a pyranometer (LI-200; LI-COR Inc.) coupled to a LI-185B Light Meter (LI-COR Inc.). The measurement of far-red fluence rate was made with and without an infra-red gelatin filter (87C; Kodak, Rochester, NY); the measurement made with this filter was subtracted from the total measurement to estimate the approximate energy below 800 nm. The fluence rates of light were varied when necessary by varying the duration of irradiation, the distance between the plants and light source or with the aid of neutral density filters made from Miracloth (Calbiochem Corp., San Diego, CA). Fluence rates for individual experiments are given in the figure legends. All manipulations of dark-grown plants were performed either in complete darkness or in light provided by a dim, green safelight as described previously (Schiff, 1972).

Plastid isolation

Plastids were isolated from the apical 3 cm of seedlings on Percoll gradients as previously described (Klein and Mullet, 1986). All manipulations were performed at 2–4°C. For quantitation of plastid number (plastids/μl of suspension volume), intact plastids were diluted and counted in a hemocytometer with a ×20 phase contrast lens.

Isolation of plastid nucleic acid

Total nucleic acid was isolated from intact plastids by phenol extraction as described previously (Mullet *et al.*, 1985). The nucleic acid was resuspended in TE buffer (10 mM Tris−HCl, pH 8.0, 0.1 mM EDTA) on a per plastid basis.

Primer extension analysis

5′ end DNA labeling was done according to Maniatis *et al.* (1982). Primer extension assays were performed as described previously (Mullet *et al.*, 1985). Labeled fragments were analyzed on 8% polyacrylamide−8.3 M urea gels (Maxam and Gilbert, 1980).

Isolation of total leaf RNA

Total nucleic acid from leaf tissue was isolated as described previously (Klein *et al.*, 1988). Following nucleic acid recovery, the nucleic acid extract was treated with DNase I (Promega Biotech Corp., Madison, WI), phenol extracted and precipitated twice. RNA concentration was calculated using one absorbance unit at 260 nm equal to 40 μg RNA/ml.

Treatment of seedlings with the transaminase inhibitor, AFPA

Barley seeds were imbibed for 1 h in distilled water. Following imbibition, seeds were transferred to a tray containing paper towels moistened with Hoagland's nutrient solution. After 2 days in complete darkness, seedlings were transplanted to 50 ml beakers containing four layers of 3 MM chromatography paper (Whatman) soaked with either 3 ml of water or 3 ml of 1 mM AFPA. Growth was continued for another 3 days in total darkness and then seedlings were either kept in darkness or transferred to an illuminated chamber (350 $\mu E/m^2/s$, fluorescent plus incandescent bulbs) for 4 h prior to tissue harvest. Seedlings were cut 1 cm above the seed and 1 cm below the leaf tip and total leaf nucleic acid extracted as described above.

In vivo uptake experiments

Approximately 30 leaves from dark-grown seedlings (upper 4 cm) were cut and immediately immersed in water and recut at the base of the stem. Seedlings were quickly placed in small vials containing 300 μl of 40 mM HEPES−KOH (pH 8.0). When appropriate, cycloheximide (20 μg/ml final concentration) or chloramphenicol (100 μg/ml final concentration) was added. Seedlings were incubated in the appropriate solutions for 1 h at 23°C in a dark chamber. The evaporative demand was increased by blowing cool air from a hair drier over the seedlings. After 1 h in the dark, the seedlings were either kept in darkness for an additional 2 h or transferred to an illuminated chamber (350 $\mu E/m^2/s$, fluorescent plus incandescent bulbs) for 2 h. When solutions became low during this period, 40 mM HEPES−KOH (pH 8.0) (plus cycloheximide or chloramphenicol as appropriate) was added to each vial. Following incubation, intact plastids were isolated and total plastid nucleic acid extracted as described above.

Acknowledgements

This work was supported by grant no. GM37987-01 from the National Institutes of Health to J.E.M.

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Received on April 20, 1989; revised on June 16, 1989