Planta (1987) 172:393–399

Planta
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Temporal and light control of plastid transcript levels for proteins involved in photosynthesis during mustard (*Sinapis alba* L.) seedling development

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Abstract. Transcript levels of four plastid genes encoding constituents of the photosynthetic apparatus were assessed in cotyledons of developing mustard (*Sinapis alba* L.) seedlings. These genes, encoding the P700 apoproteins of photosystem I, the alpha subunit of the extrinsic CF_1 moiety of the plastid ATP synthase complex, and the cytochromes *f* and b_6, have been localized and mapped previously on mustard chloroplast DNA (G. Dietrich and G. Link, 1985 Curr. Genet. **9**, 683–692). Dot blot and Northern hybridization analysis provides evidence that in dark-grown seedlings transcript levels of all four genes rise between 30 h and 72 h after sowing and thereafter fall again, pointing to the existence of an endogenous, light-independent, developmental program. In light-grown seedlings, an additional enhancement of transcript levels beyond the dark values becomes noticeable at approx. 30–36 h and then continues throughout the subsequent "light-responsive" phase until 96 h after sowing. This is consistent with a photoregulated modulation mechanism operating once "competence" has been reached. Enhanced transcript accumulation occurs following continuous illumination by either white light or (photosynthetically inefficient) far-red light thought to operate mainly through phytochrome. However, the degree of light enhancement for the transcript specifying the P700 apoproteins is higher with white light than with far-red light, implying involvement of additional photoreceptor(s) mediating this response. In addition to the endogenous gross regulation and light enhancement, a fine regulation of transcript levels seems to operate, as indicated by temporal variations of two related transcripts originating from the cytochrome-*f* gene region. The observed developmental and photocontrolled changes in specific transcript levels for photosynthesis proteins are only reflected in part by changes in total RNA content and do not appear due to light-dark differences in plastid-DNA copy number during mustard seedling development.

Key words: Chloroplast gene expression – Competence – Photosynthesis – Phytochrome – *Sinapis*.

Introduction

Mustard (*Sinapis alba* L.) seedlings have been widely used in studies aimed at defining the onset and extent of developmental control by light and to clarify the involvement of the photoreceptor(s) in this control. These studies have focussed on physiological parameters and, more recently, on the assessment of specific proteins and RNAs in response to light (for review see Schopfer 1984). In particular, chloroplast biogenesis from the proplastid stage has been found to be stringently controlled in this system by light acting through the photoreceptors phytochrome and protochlorophyllide (for review see Mohr 1984). Light control has been recently shown at the molecular level for the Q_B-protein mRNA (Link 1982, Hughes et al. 1987) and for the two subunits and cognate mRNAs of the Calvin-cycle enzyme ribulose-1,5,-bisphosphate carboxylate carboxylase/oxygenase (RuBPCase). Both the nuclear-encoded small sub-

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Abbreviations: CF_1-alpha = alpha subunit of the CF_1 moiety of the plastid ATP synthase; cyt b_6 = cytochrome b_6; cyt *f* = cytochrome *f*; P700 = P700 apoprotein of photosystem I; RuBPCase = ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); ptDNA = plastid DNA

unit and the plastid-encoded large subunit as well as their mRNAs have been found to be under phytochrome control (Oelmüller et al. 1986).

A detailed physical map of mustard plastid DNA is available (Link et al. 1981; Link 1984) and the position and orientation of many plastid genes on this map has been established by molecular cloning and hybridization techniques (Link 1981; Dietrich and Link 1985; Neuhaus and Link 1987). Among these plastid genes, several structural genes for proteins involved in photosynthesis were identified, thus allowing analyses of their expression during light-controlled seedling development. Here we report on studies in which we have assessed the steady-state concentrations of transcripts for the alpha subunit of the CF_1 moiety of the plastid ATP synthase complex (CF_1-alpha), the P700 apoproteins of photosystem I (P700), and for cytochromes *f* (cyt*f*) and b_6 (cytb_6).

Material and methods

Mustard (*Sinapis alba* L.) seeds were obtained from Asgrow Company (Freiburg-Ebnet, FRG) in 1979. Seedlings were grown at 25° C as described previously (Mohr 1966) in darkness or with continuous illumination by light from white fluorescent lamps (12000 lx ; approx. 30 $W \cdot m^{-2}$; Reiss et al. 1983) or by standard far-red light (λ_{max} at 765 nm; 3.5 $W \cdot m^{-2}$; half-band-width 120 nm; Mohr 1972).

Purification of RNA from mustard cotyledons of various developmental stages was by phenol/chloroform extractions and treatment with desoxyribonuclease I (DNase I) as described by Link (1982). The average amount of RNA extracted per cotyledon pair (RNA content) was determined by measuring the absorbance at 260 and 280 nm (1 $OD_{260} = 40$ μg ml^{-1}). For quantitative determination of plastid transcripts by using dot-blot hybridization, RNA was denatured in 10XSSC (1XSSC = 0.15 M NaCl, 0.015 M sodium citrate), 6% formaldehyde. The RNA samples (6 μg and serial dilutions) were applied to nitrocellulose (Cheley and Anderson 1984) and hybridized with ^{32}P-labelled nick-translated (Rigby et al. 1977) plastid DNA (ptDNA) fragments (Maniatis et al. 1982). Relative concentrations of plastid transcripts were derived from the amounts of probe DNA hybridized (as measured by scintillation counting). Values obtained for 10 μg cotyledon RNA (in cpm above background; by extrapolation from the measured values within the linear range of the DNA/RNA hybridization curves) were multiplied with the total RNA content per cotyledon pair (in μg) to give the transcript level per cotyledon pair. Northern hybridization analysis (Thomas 1980) of electrophoretically separated plastid RNAs was as described by Maniatis et al. (1982).

Desoxyribonucleic acid was prepared from seeds and cotyledons in the same way as RNA, except for replacing the DNase I step by incubation with DNase-free RNase A (20 μg per ml; 15 min at 37° C). Samples of DNA (10 μg) were electrophoretically separated on 0.7% agarose gels, blotted to nitrocellulose (Southern 1975), and hybridized to nick-translated ptDNA fragments using standard procedures (Maniatis et al. 1982).

The following gene-specific mustard ptDNA fragments were used as probes: BamHI/BamHI 1.9 (intragenic) within plasmid pSA244-B1.9, a subclone of pSA244 in pUC13, as P700 probe; EcoRI/EcoRI2.6 and HindIII/HindIII0.8 (intragenic) within plasmid pSA364-E2.6, a subclone of pSA364, as CF_1-alpha probes; EcoRI/EcoRI1.75 and EcoRI/HindIII0.7 (intragenic) in pSA110-E1.75, a subclone of pSA110, as cytb_6 probes; EcoRI/EcoRI2.7 and ClaI/ClaI0.25 (intragenic) in pSA120-E2.7, a subclone of pSA120, as cyt*f* probes (Dietrich and Link 1985); the 1.0-kb PstI/EcoRI fragment of plasmid pSA452 as Q_B-protein gene probe (Link 1981).

Statistics. Values given in Fig. 1 are mean values of three experiments with SEs of approx. 10%.

Results

Figure 1 shows the RNA content (total RNA extracted) and steady-state levels of the plastid transcripts for CF_1-alpha, P700 apoproteins, and cyt*f* in cotyledons of mustard seedlings at 24 to 96 h after sowing. During this time period, transcript levels of all three plastid genes measured increase almost simultaneously in both light-grown and dark-grown seedlings, with the onset of accumulation occurring at 30 to 36 h after sowing. Transcript levels are maximal at approx. 72 h and then decrease again in older cotyledons at 84 h and 96 h after sowing (Fig. 1 B–1 D). In each case the time-course of transcript accumulation resembles the time-course of total RNA accumulation (Fig. 1 A). However, there is only a three- to fourfold increase in total cotyledon RNA during seedling development from 24 to 72 h, while specific plastid transcript levels increase as much as one to two orders of magnitude during this time period.

Transcripts of the three plastid genes accumulate to higher peak levels in light-grown than in dark-grown seedlings at 72 h after sowing (Fig. 1 B–1 D). Although this partly reflects the somewhat higher total RNA content in cotyledons of light-grown seedlings (Fig. 1 A), the ratio of peak values in light-grown versus dark-grown seedlings is higher for levels of CF_1-alpha, P700 and cyt*f* transcripts than for total cotyledon RNA.

In attempts towards defining photoreceptor(s) involved in the enhanced accumulation of total RNA and of the plastid transcripts in light-grown seedling, two different light conditions were applied. Seedlings were grown either in white light known to promote photosynthesis as well as non-photosynthetic morphogenetic processes mediated through phytochrome (Schmidt et al. 1987), or alternatively, continuous irradiation was applied by standard far-red light, which largely prevents chlorophyll formation (photosynthesis), but activates phytochrome-dependent physiological responses (Mohr 1972). Total cotyledon RNA (Fig. 1 A) as

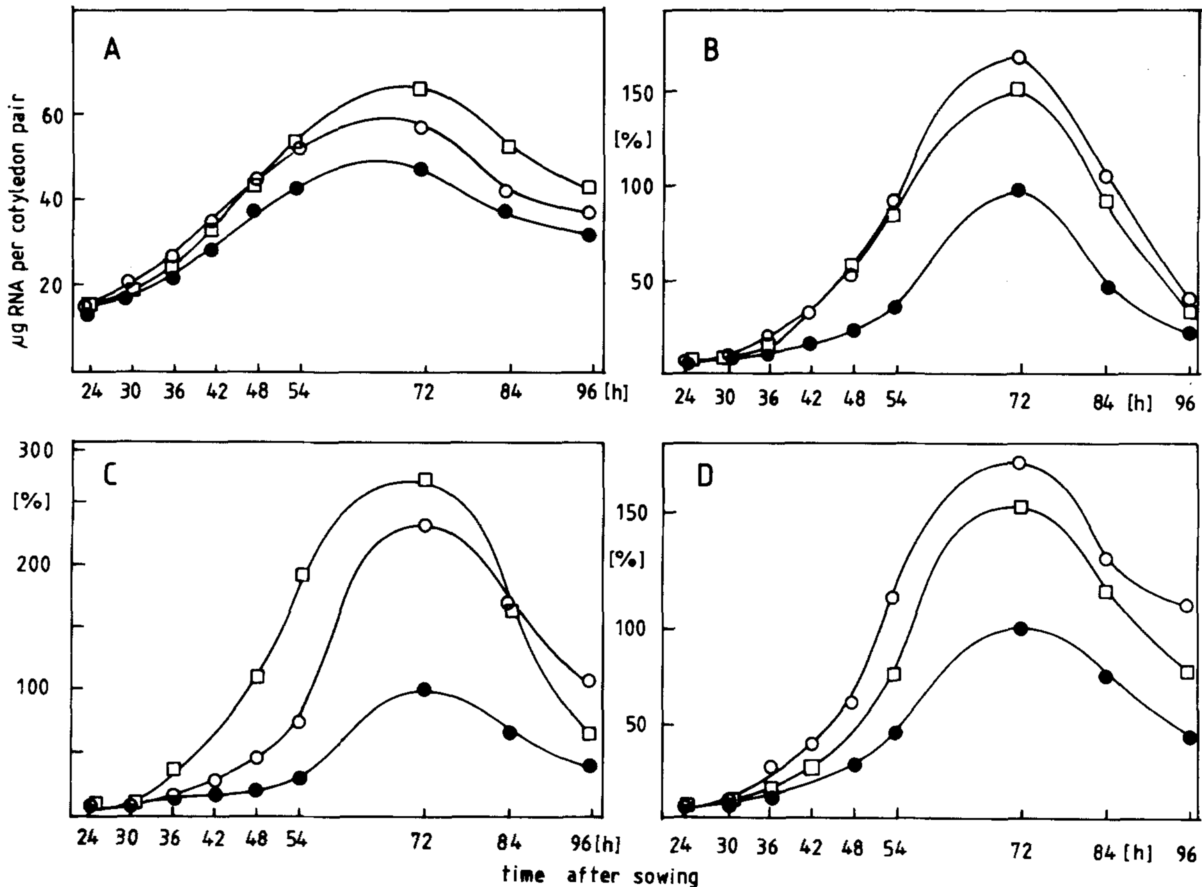

Fig. 1 A–D. Quantification of total RNA and plastid transcript levels in mustard cotyledons. **A** RNA content (total RNA extracted) per cotyledonpair. **B–D** Transcript levels determined by dot-blot-hybridization analysis (see *Material and methods*) for CF_1-alpha (**B**) P700 apoproteins (**C**), and cyt*f* (**D**). Seedlings were grown under white light (□), continuous far-red light (○), or in darkness (●) for 24 to 96 h

well as P700 transcript levels (Fig. 1 C) accumulate to higher values in seedlings grown under white light than in those grown under far-red light. Accumulation kinetics of the transcripts for CF_1-alpha (Fig. 1 B) and cyt*f* (Fig. 1 D) on the other hand show that continuous far-red is at least as effective as white light in enhancing peak levels of these transcripts at 72 h after sowing. Phytochrome thus appears to be involved in the light regulation of both total cotyledon RNA and specific transcript levels for CF_1-alpha, P700 apoproteins, and cyt*f*, although to a different extent. However, these hybridization results also emphasize the additional involvement of other controls, likely operating at shorter wavelengths and possibly related to the onset of photosynthesis (Oelmüller and Mohr 1984; Schmidt et al. 1987), in the photomorphogenesis of the mustard seedling.

Plastid transcript levels during mustard seedling development were further characterized by Northern blot analysis (Fig. 2), allowing direct visualization of distinct RNA species. These transcripts include those investigated by dot-blot analysis (Fig. 1) and, in addition, the cytb_6 transcript (Dietrich and Link 1985). The 2.2 kb mRNA for CF_1-alpha (Fig. 2 E, F) and the 5.1-kb P700 (primary) transcript (Fig. 2 G, H) both reveal a time-course which is in agreement with the results of dot-blot hybridization (Fig. 1), i.e. their relative levels (in equal amounts of total RNA used at each time point) increase considerably between 36 and 72 h both in the light and in the dark. A similar time-course is also found for the 1.9-kb cytb_6 mRNA (Fig. 2 A, B) as well as for the 1.4-kb cyt*f* transcript and the 1.7-kb transcript, which probable represents a cyt*f* mRNA precursor (Dietrich and Link 1985; Fig. 2 C, D). Both the 1.7-kb and 1.4-kb transcript levels decline by similar proportions with RNA from dark-grown seedlings representing the later stages of seedling development 84 and 96 h after sowing (Fig. 2 C). With RNA from light-grown seedlings, however, relative levels of the 1.7-kb cyt*f*-related RNA species decrease, while levels of the mature 1.4-kb cyt*f* mRNA ap-

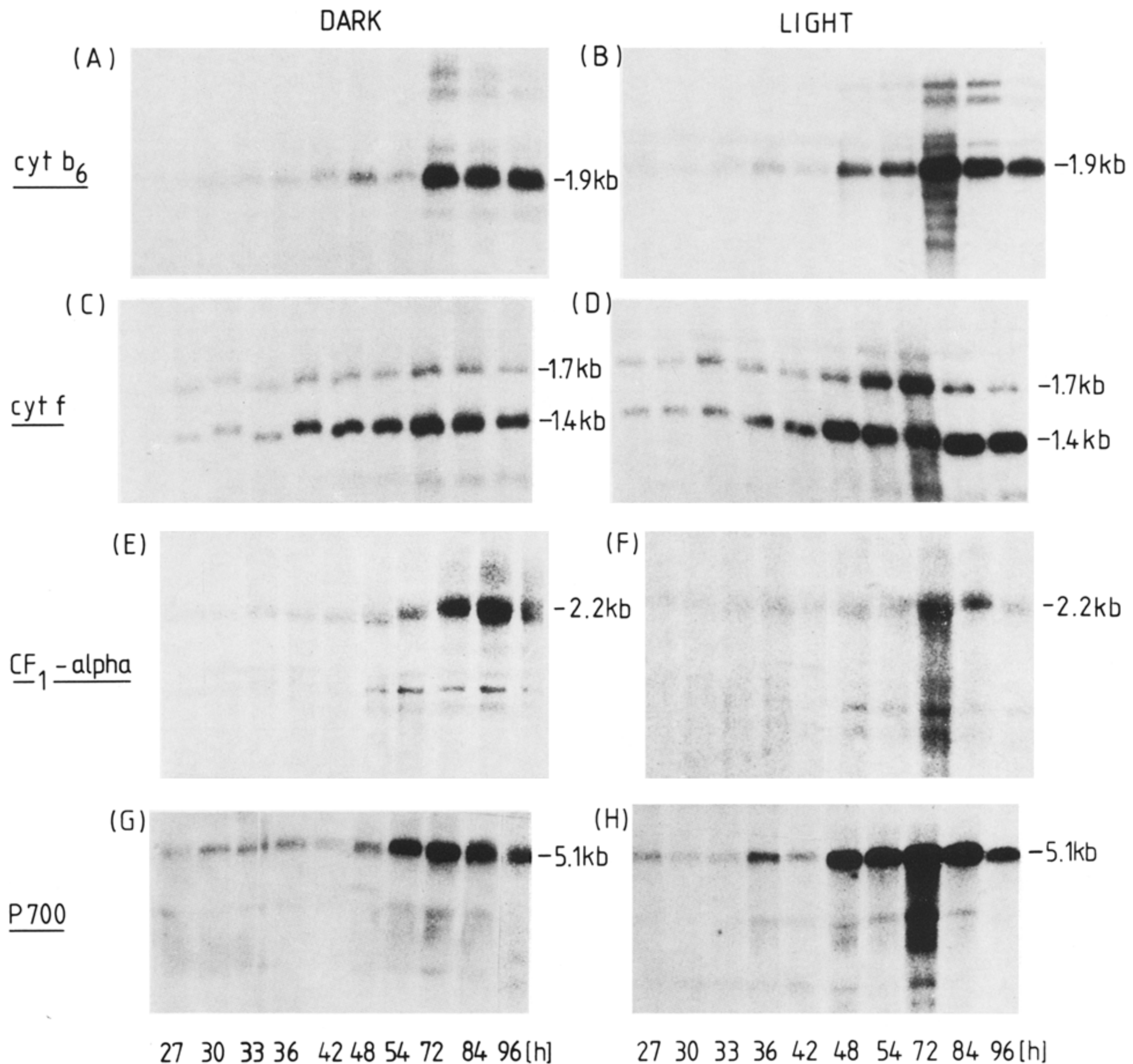

Fig. 2 A–H. Northern hybridization analysis of specific transcript levels during mustard seedling development. Equal amounts of total cotyledon RNA (10 μg) were electrophoretically separated, transferred to nitrocellulose (Thomas 1980), and hybridized with gene-specific ptDNA probes (see *Material and methods*). **A**, **B** cytb_6, **C**, **D** cyt*f*, **E**, **F** CF_1-alpha, **G**, **H** P700 apoprotein transcripts. Seedlings were grown either in the dark (*left-hand panels*) or in continous far-red light (*right-hand panels*) for 24 to 96 h after sowing

pear to remain largely constant (Fig. 2 D). As the total RNA content per cotyledon decreases during the late phase of seedling development (Fig. 1 A), absolute levels of both transcripts decline, but to a different extent. This differential hybridization of the two related transcripts might point to light/dark differences in the processing pathway leading to mature cyt*f* mRNA. The time-course of cytb_6 mRNA (Fig. 2 A, B) appears very similar to that of cyt*f* mRNA during late seedling development.

To approach the problem of whether or not the observed light/dark differences in specific transcript levels might be related to differences in gene copy number in light-grown versus dark-grown seedlings, the plastid DNA content of cotyledons was assessed (Fig. 3). A hybridization approach involving total cellular DNA was chosen, as this obviates the need for quantitative isolation of plastids and ptDNA from small amounts of tissue: restriction-enzyme-digested DNA was electrophoresed in agarose gels, blotted (Southern 1975), and hybridized with a defined plastid-specific probe. The chosen probe represented a region of the Q_B-protein

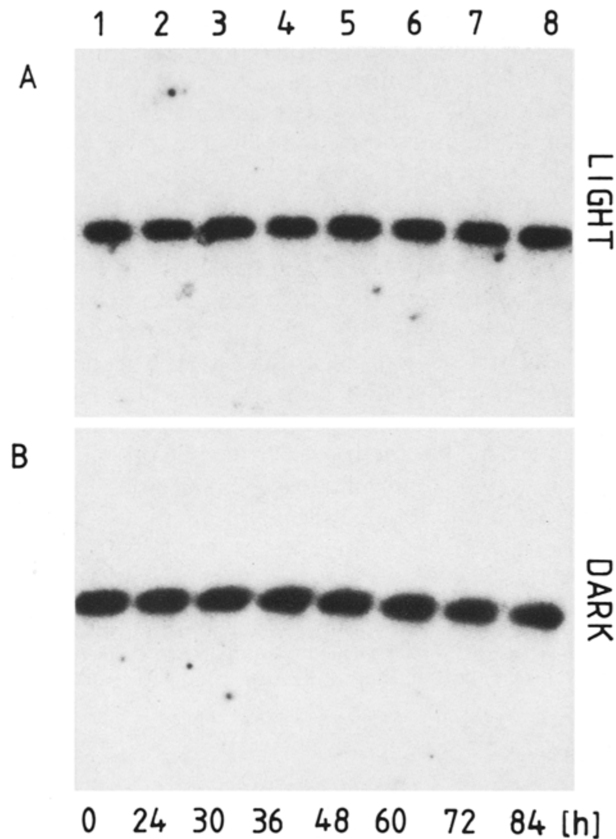

Fig. 3 A, B. Assessment of ptDNA copy number during mustard seedling development. Total cellular DNA samples (10 μg) were digested with restriction endonuclease PstI. DNA fragments were then electrophoretically separated in 0.7% agarose gels, blotted (Southern 1875), and hybridized with a DNA probe containing Q_B-protein DNA sequences (Link and Langridge 1984; see *Material and methods*). The DNA was extracted from seeds (*Lane 1*) and cotyledons (*Lanes 2–8*) of 24- to 84-h-old mustard seedlings grown either under white light (**A**) or in darkness (**B**)

gene, which had been previously characterized as to its precise map position and nucleotide sequence (Link 1981; Link and Langridge 1984) and is known to give rise to transcript levels with the highest light/dark difference among all plastid genes investigated thus far (Link 1982; Link 1984). Hybridization bands of comparable intensity are generated both with seed DNA and cotyledon DNA from seedlings 24–84 h after sowing. At each time-point the total DNA content (i.e. extractable DNA) per cotyledon pair is approx. 15% of the total nucleic acid content (data not shown), indicating that DNA accumulates with kinetics similar to those shown for RNA (Fig. 1 A). Plastid DNA content per cotyledon thus likewise appears to increase during early mustard seedling development up to 72 h and then to decrease again between 72 and 96 h. Hybridization bands of similar intensity are generated with DNA from dark-grown seedlings (Fig. 3 B) and from light-grown seedlings (Fig. 3 A). Hence, there is no evidence for light/dark differences in ptDNA copy number per cotyledon in the mustard seedling.

Discussion

In the present work we have investigated developmentally-regulated and light-regulated changes of transcript levels in mustard seedlings for constituents of three functional complexes of the photosynthetic apparatus, i.e. for the P700 apoproteins of the photosystem I reaction center, for cytochromes f and b_6, and the alpha subunit of the extrinsic CF_1 moiety of the chloroplast ATP synthase complex (CF_1-alpha). A similar transcript analysis, providing complementary information, was recently carried out with the psbA gene encoding the M_r-32000 herbicide-binding Q_B-protein of photosystem II (Hughes et al. 1987). These transcripts for photosynthesis proteins show several common features with regard to their mode of accumulation.

(i) The transcript accumulation at 30–72 h, both in light-grown and dark-grown seedlings, followed by a decrease at later stages, reflects an endogenous developmental program of plastid gene expression, which appears to be independent of light. The onset of this temporally regulated expression seems to be synchronized for all plastid genes studied so far, including groups of physically distant genes (Dietrich and Link 1985). An almost simultaneous initial rise in transcript levels at 30–36 h after sowing is observed for the genes encoding P700 apoproteins, CF_1-alpha, cyt$f$ and cytb_6 (see *Results*), for the genes encoding RuBPCase large subunit (Oelmüller et al. 1986) and the M_r-32000 Q_B-protein gene product (Hughes et al.1987), for the putative gene encoding the M_r-47000–51000 P680 apoprotein of photosystem II as well as for several as yet unidentified plastid genes (data not shown).

(ii) Light neither influences the onset nor the time point of maximal transcript accumulation, but rather appears to result in a multiplicative enhancement of transcript levels controlled by the endogenous program. The absolute degree of light enhancement seems to vary for each transcription unit studied, thus representing a gene-specific feature, not fully accounted for by the observed general light/dark difference of total cotyledon RNA content (Fig. 1 A). It is interesting to note that the extent to which transcript levels decrease between

72 and 96 h seems to vary (Fig. 1). The Q_B-protein transcript was shown to accumulate even further during this phase in the light but not in the dark (Hughes et al.1987). These observations might indicate light/dark differences in transcript stability during late seedling development.

(iii) Light enhancement of plastid transcript levels during mustard seedling development does not appear to be the consequence of light/dark differences in plastid DNA copy number (Sasaki et al. 1984) per cotyledon, providing support to the notion that the observed transcript levels might rather be caused by differential gene expression at the level of transcription and-or RNA processing.

(iv) The RNA levels of the genes studied here respond differently to the two irradiation programs used. Transcript levels for CF_1-alpha and cyt*f* are enhanced at least to the same extent by (photosynthetically inefficient) continuous far-red light as by white light, implying phytochrome involvement in eliciting these responses (Mohr 1972). This is in agreement with the results of red/far-red pulse irradiation and reversion experiments (data not shown). Conversely, the P700 transcript as well as the plastid ribosomal RNAs and the Q_B-protein transcript (data not shown) accumulate to higher levels in white light than in far-red light. Hence, this further substantiates the notion that other receptor(s), e.g. related to chlorophyll biosynthesis and-or operating at shorter wavelengths (Oelmüller and Mohr 1984), seem to be required for optimal expression of at least several plastid genes in this system.

How might transcript levels of (adjacent and physically distant) plastid genes be regulated during photomorphogenesis? The similar time-courses of various transcripts point to the presence of an endogenous program providing, light-independent, coordinated expression of many plastid genes during seedling development. This could be brought about by a single general mechanism such as modification of the transcription apparatus or changed topology of the ptDNA (Stirdivant et al. 1985). Synthesis or activation of effectors resulting in such a general switch could be expected to be transient events. It is interesting to note that a split plastid $tRNA^{Lys}$ gene with an intron potentially encoding a protein gives rise to a transcript with transient peak levels during early mustard seedling development (Hughes et al. 1987). This would be consistent with the idea that endogenous developmental "competence" (Mohr 1984) is a prerequisite for subsequent modulation (enhancement, possibly decrease) of transcript levels by light. Such modulation appears to involve more than one photoreceptor, implying that there might be more than one single mechanism to achieve light-mediated changes in plastid gene expression. The different extent of light modulation observed for various plastid transcripts indicates that either these genes differ in their responsivity status with regard to light modulation at the level of transcription or that post-transciptional regulation is involved or both. Post-transcriptional fine regulation appears to play a role, as e.g. indicated by the variable levels of the related 1.4-kb and 1.7-kb transcripts of the cyt*f* gene region (Fig. 2 C, D). Finally, each of the regulatory mechanisms envisaged might be mediated by interactions of the plastid with the nucleo-cytoplasmic genetic system (Reiss and Link 1985; Oelmüller et al. 1986).

We are grateful to M. Krenz and C. Wittig for expert technical assistance and to J.E. Hughes and R. Oelmüller for helpful discussion. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 206 and Schwerpunkt Molekularbiologie der Pflanzen) and Fonds der Chemischen Industrie, FRG.

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Received 5 March, accepted 20 May 1987