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Regulatory factors involved in gene expression (subunits of ribulose-1,5-bisphosphate carboxylase) in mustard (*Sinapis alba* L.) cotyledons

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Abstract. Phytochrome-controlled appearance of ribulose-1,5-bisphosphate carboxylase (RuBPCase) and its subunits (large subunit LSU, small subunit SSU) was studied in the cotyledons of the mustard (*Sinapis alba* L.) seedling. The main results were as follows: (i) Control of RuBPCase appearance by phytochrome is a modulation of a process which is turned on by an endogenous factor between 30 and 33 h after sowing (25° C). Only 12 h later the process begins to respond to phytochrome. (ii) The rise in the level of RuBPCase is the consequence of a strictly coordinated synthesis de novo of the subunits. (iii) While the levels of translatable mRNA for SSU are compatible with the rate of SSU synthesis the relatively high LSU mRNA levels are not reflected in the rates of in-vivo LSU or RuBPCase syntheses. (iv) Gene expression is also abolished in the case of nuclear-encoded SSU if intraplastidic translation and concomitant plastidogenesis is inhibited by chloramphenicol, pointing to a "plastidic factor" as an indispensable prerequisite for expression of the SSU gene(s). (v) Regarding the control mechanism for SSU gene expression, three factors seem to be involved: an endogenous factor which turns on gene expression, phytochrome which modulates gene expression, and the plastidic factor which is an indispensable prerequisite for the appearance of translatable SSU mRNA.

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Abbreviations: CAP = chloramphenicol; cFR = continuous far-red light; LSU = large subunit of RuBPCase; NADP-GPD = NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13); Pfr = far-red-absorbing form of phytochrome; pSSU = precursor of SSU; RuBPCase = ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39); SSU = small subunit of RuBPCase

Key words: Gene expression – Phytochrome – Plastidic factor – Ribulose-1,5-bisphosphate carboxylase – *Sinapis*.

Introduction

Plastidogenesis in higher plants is characterized by its light dependency: the appearance of many plastidic proteins is controlled by light via phytochrome (Mohr 1984; Tobin and Silverthorne 1985). Moreover, plastid development depends on the coordinated expression of nuclear and plastidic genes. In recent efforts to understand the control mechanisms, RuBPCase, the enzyme responsible for CO_2 fixation in photosynthesis, has attracted particular interest (see Inamine et al. 1985 for references to pertinent literature). The enzyme RuBPCase is a multimeric protein comprised of eight large subunits (LSU) and eight small subunits (SSU) (Baker et al. 1975). The enzyme is localized in the plastid, but the plastid genome encodes only the LSU. The genetic information of the SSU is nuclear-coded. The SSU is synthesized in the cytoplasm as a precursor (pSSU), which is processed and transported into the plastid where it combines with the LSU to form the active enzyme (see Inamine et al. 1985 for references to pertinent literature).

Previous studies using pea seedlings have shown that the steady-state level of mRNA coding for the SSU of RuBPCase increases strongly upon exposure of the plants to light (Smith and Ellis 1981; Thompson et al. 1983; Coruzzi et al. 1984). This appears to be due to a specific increase in

transcription since the copy number of the SSU gene(s) in the nucleus was not significantly affected by light (Sasaki et al. 1986). Run-off transcription experiments using isolated pea or *Lemna* nuclei (Gallagher and Ellis 1982; Silverthorne and Tobin 1984) indicate that light increases the steady-state levels of SSU mRNA by increasing the transcription of the SSU gene(s).

The level of LSU mRNA also increases after exposure of pea plants to light, but in this case there appears to be a correlation between the rate of increase in LSU mRNA and an increase in plastid genome copy number (Thompson et al. 1983; Sasaki et al. 1984, 1986).

In mustard seedling cotyledons, accumulation of RuBPCase and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GPD) is known to be controlled by phytochrome (Brüning et al. 1975). The young mustard seedling does not synthesize RuBPCase or NADP-GPD, and only traces of seed-borne enzyme activity can be detected before 36 h after sowing at 25° C. An important finding in the present context was that for the appearance of both enzymes it does not matter whether the light which activates phytochrome is given from the time of sowing, from 24 h after sowing, or only from 36 h after sowing onwards (Brüning et al. 1975). This was interpreted to indicate that the mustard seedling is not "competent" to respond to phytochrome by synthesis of NADP-GPD or RuBPCase before 36 h after sowing (25° C) (Mohr 1983). Other enzymes in the mustard cotyledons, not related to photosynthesis, can be induced by phytochrome much earlier in the course of development. There is obviously a temporal pattern of inducibility of enzymes by the far-red-absorbing form of phytochrome (Pfr). The specification of this "temporal pattern of competence to Pfr" is not influenced by phytochrome (Mohr 1983).

In a previous paper (Oelmüller and Mohr 1984) we have shown that an increase in the activity of NADP-GPD becomes detectable at the same time in dark-grown material as in the seedling kept in continuous far-red light (cFR) (i.e. kept continuously, from the time of sowing, under the strong action of phytochrome). During the first 6 h after the onset of enzyme synthesis the increase of NADP-GPD activity in FR is approx. 2.7 times the increase in the dark. Thus, the light effect is multiplicative, indicating a modulation of gene expression by phytochrome rather than an induction proper? The rate of NADP-GPD accumulation decreases in darkness beyond 48 h while it steeply increases in the light. This indicates that accumulation of NADP-GPD crucially depends on phytochrome action from 48 h onwards.

In the present paper we describe the appearance of RuBPCase and its subunits in the cotyledons of the mustard seedling. The following questions are addressed: (1) Is control of RuBPCase synthesis by phytochrome a modulation or an induction proper? In the case of modulation, are the time courses of appearance of RuBPCase in light and darkness multiplicatively related as described previously for NADP-GPD (Oelmüller and Mohr 1984)? (2) Are measurements of the pertinent mRNA levels compatible with the rates of synthesis of the subunits? (In pea the situation with LSU mRNA is not clear, see Inamine et al. 1985.) (3) Is accumulation of RuBPCase the result of a coordinated synthesis of LSU and SSU, including temporal coordination with regard to responsiveness to phytochrome? (4) Is gene expression abolished in the case of nuclear-encoded SSU if intraplastidic translation is inhibited and plastidogenesis impaired by the application of chloramphenicol (CAP)?

Material and methods

Seeds of white mustard (*Sinapis alba* L.) were obtained from Asgrow Company (Freiburg-Ebnet, FRG) in 1979. Selection of the seeds, germination and growth conditions (25±0.5° C) were as described previously (Mohr 1966).

For light treatment, a standard far-red light source (3.5 $W \cdot m^{-2}$, Mohr 1966) was used. This kind of light does not cause significant protochlorophyll(ide) → chlorophyll(ide) photoconversion, while phytochrome-mediated responses are strongly potentiated (Mohr 1972).

Ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) activity was assayed at pH 8.2 by measuring the acid-stable radioactivity produced in the reaction between ribulose-1,5-bisphosphate and $NaH^{14}CO_3$ at 25° C according to Frosch et al. (1979).

For in-vivo labelling of total protein, 20 pairs of cotyledons were shaken immediately after removal from the seedling in 2 cm^3 of a radioactive [^{3}H]leucine solution ($15 \cdot 10^{10}$ Bq per pair of cotyledons) for 30 min in green safelight, the cotyledons were washed four times with a large excess of distilled water and frozen in liquid nitrogen. The cotyledons were ground with 1 g quartz sand, 0.5 g Dowex 1×2 and 4 cm^3 of extraction buffer (100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl, pH 7.4, 150 mM NaCl, 5 mM ethylene diaminetetraacetic acid (EDTA) and the homogenate clarified by centrifugation (20 min, 39,000 · *g*). A 100-mm^3 aliquot of the supernatant was used to determine the radioactivity incorporated into the protein fraction by collecting the trichloroacetic-acid precipitate on GF/C Whatman filters (Whatman, Springfield Mill, Kent, UK).

For immunoprecipitation of LSU and SSU, 100 mm^3 of the supernatant was incubated with 50 mm^3 of RuPBCase antiserum, incubated for 1 h at 25° C and at 4° C overnight. *Staphylococcus aureus* cells (50 mm^3) were used to achieve the precipitation of the antibody-antigen complex. Washing of the precipitate, separation of the immunoprecipitated polypeptides by

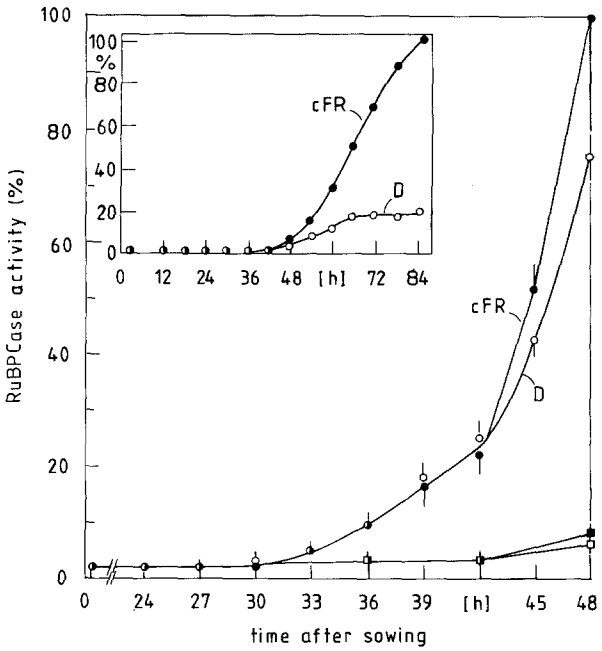

Fig. 1. Time course of RuBPCase activity in mustard cotyledons. Seedlings were either kept in darkness (D, ○, □) or in cFR (●, ■). The *inset* (adopted from Brüning et al. 1975) illustrates the strong long-term effect of cFR (%), the highest value of each graph was taken as 100%. ◑, seedlings grown on water; ◧, seedlings grown on a watery chloramphenicol solution (20 $\mu g \cdot cm^{-3}$). Values are means of 10 independent experiments. Bars indicate estimates of SE

polyacrylamide gel electrophoresis (PAGE) and fluorographic detection of the radioactively labelled products were as described by Oelmüller and Mohr (1986). Immunoprecipitates of an equal amount of radioactively labelled protein were separated on each lane.

For quantitative determination of the radioactively labelled SSU and LSU, 1 μg of purified RuBPCase (Goldthwaite and Bogorad 1971) was added to 20 mm^3 of probe buffer (2 cm^3 10% sodium dodecyl sulfate (SDS), 1.25 cm^3 0.5 M Tris-HCl, pH 6.8, 0.5 cm^3 mercaptoethanol, 1 cm^3 glycerol). The subunits (SSU and LSU) were visualized by precipitating the SDS-protein bands with 0.1 M KCl in a coldroom, and the bands were cut out of the gel and solubilized with 100 mm^3 of Soluene 20 before determination of radioactivity. The amount of antiserum used for the immunoprecipitation did not limit precipitation up to the highest antigen concentrations used.

Isolation of total RNA and in-vitro translation of the RNA by a protein-synthesizing system prepared from reticulocytes in the presence of [^{35}S]methionine was performed as described by Oelmüller and Mohr (1986). Quantification of the in-vitro translation products was performed as described by Suissa (1983).

For hybridization analysis, an equal amount of RNA from mustard cotyledons were denaturated in 50% formamide, 6% formaldehyde and 10 mM Mops (3(N-morpholino)propanesulfonic acid) pH 7, and applied to nitrocellulose according to Kafatos et al. (1979) or was electrophoretically separated in 1.2% agarose gels containing 6% formaldehyde, 20 mM Mops, 5 mM sodium acetate and 1 mM EDTA (Lehrach et al. 1977) and then transferred to nitrocellulose (Thomas 1980). For detection of LSU mRNA the nitrocellulose sheets were hybridized to a nick-translated, gene-specific ptDNA fragment (EcoRI/Hind III-1.0) cloned in plasmid pSA204 (Link 1981).

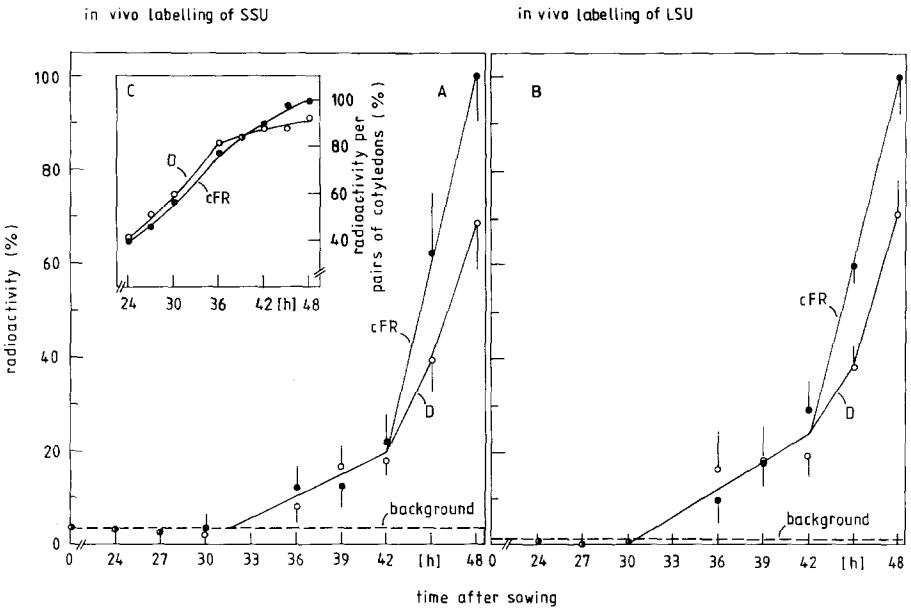

Fig. 2. Incorporation of radioactivity in SSU (14000 Da, **A**) and LSU (55000 Da, **B**) during a 30-min incubation of mustard seedlings cotyledons in a [^{3}H]leucine solution. The cotyledons were harvested at the time indicated on the abscissa immediately before incubation. The radioactive labelling of total protein (per pair of cotyledons) is shown in the inset (**C**). Immunoprecipitations of equal amounts of radioactively labelled protein were separated by polyacrylamide gel electrophoresis and the radioactivity in the SSU and LSU bands determined as described in *Material and methods*. ●, far-red-light-grown material; ○, dark-grown material. 100% = radioactive labelling of SSU and LSU in 48 h far-red-light-grown material. Values are means of three independent experiments. Bars indicate estimates of SE. Note breaks in axes

Results

Effect of light (cFR) on the time course of RuBPCase activity. A small amount of enzyme activity is always detectable, even in the seed. This level remains unchanged until 30 h after sowing (Fig. 1). Between 30 and 33 h after sowing, additional enzyme activity appears. However, a light effect is only detectable from 42 h after sowing onwards. The effect of cFR is only modest at the beginning; only beyond 48 h is a strong promotive effect observed relative to dark controls. We conclude that cFR (operating through phytochrome) positively modulates a process which occurs in absolute darkness. However, the fact that RuBPCase accumulation ceases in darkness around 66 h while it continues with a high rate in cFR indicates that enzyme synthesis crucially depends on phytochrome action from 66 h onwards.

Response to the modulating factor, phytochrome, appears 12 h later than the rise of RuBPCase activity. We conclude that an endogenous *inductive* factor turns on gene expression between 30 and 33 h after sowing, and only 12 h later does the *modulating* light factor (operating via phytochrome) come into play.

Recently it was postulated that the phytochrome-controlled appearance of RuBPCase depends on a "plastidic factor" (Oelmüller et al. 1986). If intraplastidic translation and, concomitantly, plastid development are impaired by the application of 20 $\mu g \cdot ml^{-1}$ CAP, given from the time of sowing, the appearance of RuBPCase activity is inhibited in both light and darkness (Fig. 1) even though development of the seedling is normal (Oelmüller et al. 1986). An appreciation of these findings requires measurements of nuclear-encoded SSU mRNA (see below).

Synthesis of the subunits of RuBPCase. The increase in the activity of RuBPCase is the result of increased synthesis of both subunits of the enzyme. This was shown by measuring the rates of synthesis of SSU and LSU in 30-min in-vivo labelling experiments with [^{3}H]leucine and immunoprecipitation with antiserum raised against the native RuBPCase (Fig. 2). The results indicate that up to 30 h after sowing there is no synthesis of either subunit. This result is supported by the observation that a Western blot does not reveal SSU or LSU (data not shown). Synthesis de novo of SSU and LSU is readily measured from approx. 33 h after sowing onwards in light- as well as in dark-grown material. The rate of synthesis is affected by cFR from 42 h onwards. A comparison of the accumulation curve for RuBPCase (Fig. 1) with the time courses of the rate of synthesis of both subunits leaves no doubt that the rise of enzyme activity is caused by synthesis de novo of the subunits and that synthesis of the subunits is highly coordinated, at least up to 48 h after sowing.

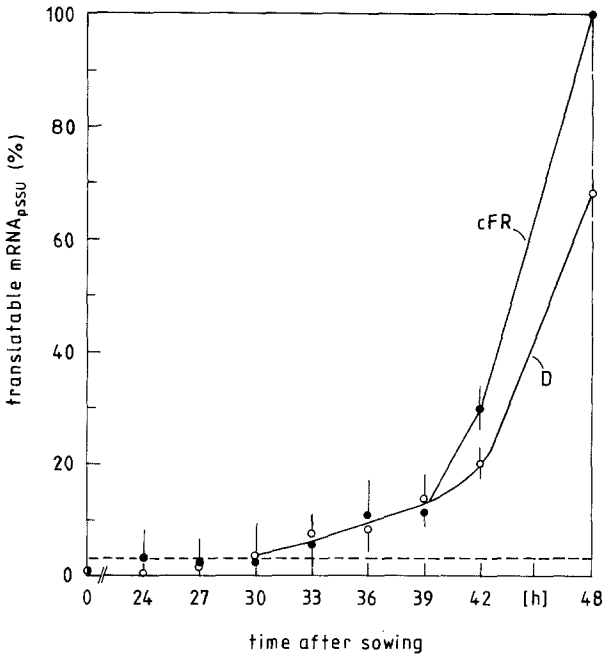

Fig. 3. Time course of the level of translatable mRNA of pSSU in cotyledons of far-red-light- (●) and dark-grown (○) mustard seedlings. Equal amounts of total RNA from cotyledons of different ages (*abscissa*) were used for in-vitro translation. For immunoprecipitation the same amount of radioactively labelled protein was used. 100% = radioactively labelled pSSU immunoprecipitate from seedlings grown for 48 h in continuous far-red (FR) light. Values are means of four independent experiments. Bars indicate estimates of SE

Time courses of mRNA levels. The time course of the level of translatable mRNA for pSSU (Fig. 3) is compatible with the rate of synthesis of SSU (see Fig. 2): up to 39 h after sowing there is no significant light effect whereas at 42 h the mRNA levels seem to differ in light and dark. In the case of LSU, in-vitro translation could not be applied since reproducible isolation of plastidic RNA from early plastid stages was not feasible. In-vitro translation of total RNA does not lead to significant amounts of LSU since plastid RNA cannot compete with nuclear RNA.

In order to compare the rate of LSU synthesis in situ (Fig. 2B) with the amount of LSU mRNA the steady-state level of LSU mRNA was measured by dot-blot hybridisation (Fig. 4A, B). Even in the seed a considerable amount of hybridizable material was detected, corresponding to a stable 1.5 kb LSU-transcript as shown by hybridisation analysis

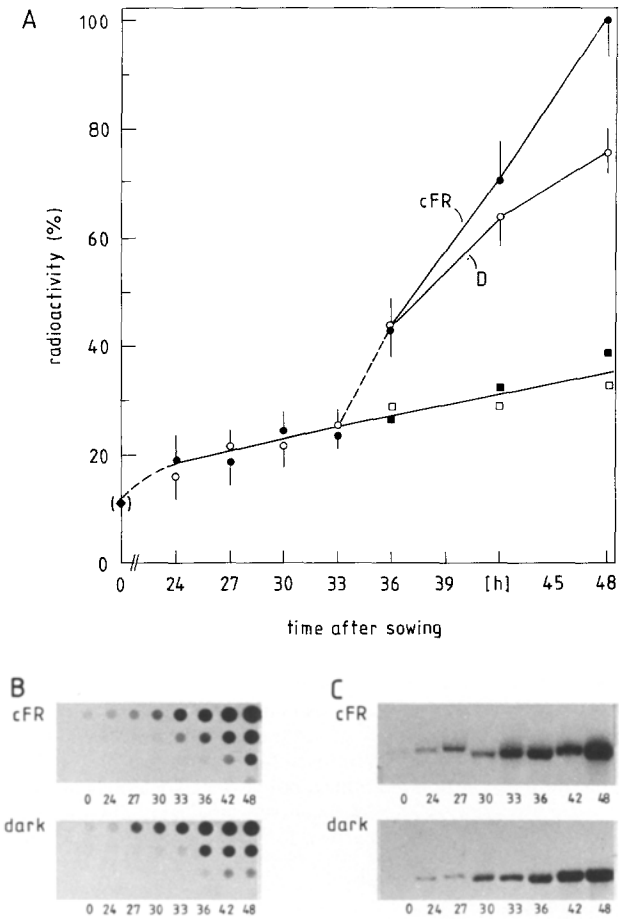

Fig. 4A–C. Time course of the amount of hybridizable mRNA for LSU in mustard cotyledons. Equal amount of total RNA from cotyledons of different ages were dotted onto nitrocellulose and hybridized to a gene-specific ^{32}P-labelled DNA-fragment of plasmid pSA204 (Link 1981). After autoradiography the dots were cut out and the radioactivity determined by liquid scintillation. **A** Quantitative determination of hybridizable mRNA (*dot blot*) for the LSU in the cotyledons of dark-grown (D, o, □) and cFR-grown (●, ■) mustard seedlings. Representative autoradiography of dot blots (**B**) and Northern analysis (**C**). o, ●, seedlings grown on water; □, ■, seedlings grown on a watery chloramphenicol solution (20 μg·cm^{-3}); ◆, amount of LSU mRNA in the seed. The values with bars are means of three independent experiments. Bars indicate estimates of SE

Table 1. Amount of translatable mRNA for the pSSU and hybridizable mRNA for the LSU in 72-h-old dark (D)- and cFR-grown mustard seedlings. The amount of mRNA of dark-grown seedlings was taken as 1.0. Results from four independent parallel experiments, estimates of SE approx. 10%

Treatment	SSU	LSU
72 h D	1.0	1.0
72 h cFR	5.9	2.1

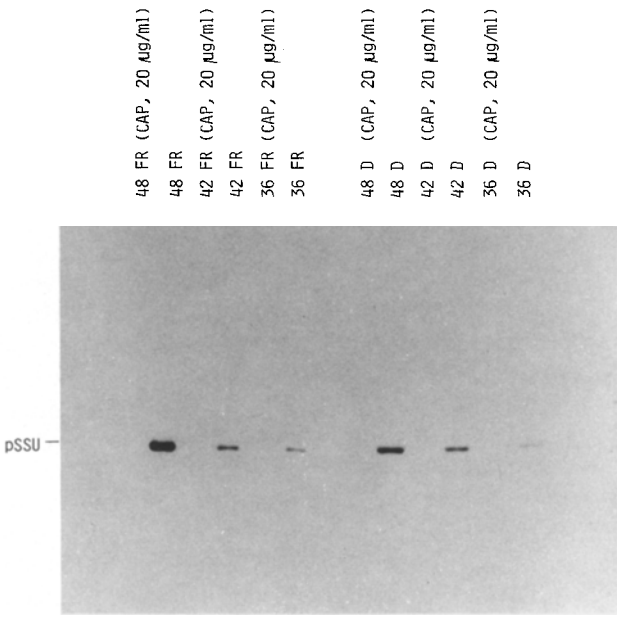

Fig. 5. Fluorogram showing the amount of translatable pSSU-mRNA at 36, 42 and 48 h after sowing, in cFR-grown and dark-grown (D) seedlings in the presence or absence of chloramphenicol (CAP, 20 μg·cm^{-3})

of electrophoretically separated RNA from mustard cotyledons (Fig. 4C). Until 30 h after sowing this LSU mRNA is not translated into a stable LSU protein (compare Figs. 2B and 4A). As far as response to light is concerned the data on LSU synthesis (Fig. 2B) and LSU mRNA level (Fig. 4A) are compatible: light comes into play only around 42 h after sowing while the rise in mRNA levels and onset of synthesis de novo of subunits and enzyme occur approx. 12 h earlier.

When seedlings were grown on 20 μg·cm^{-3} CAP solution, no sudden rise of LSU mRNA was found in dark- or in cFR-grown material. Apparently CAP inhibits not ony intraplastic translation but also accumulation of LSU mRNA, the latter effect probably being a consequence of inhibited plastid development (Oelmüller et al. 1986).

Table 1 compares the steady-state levels of SSU mRNA and LSU mRNA isolated from cotyledons of 72-h-old dark- and far-red-light-grown seedlings. The data show that the light-mediated increase in mRNA level is much greater in the case of SSU mRNA and that the strong induction of RuBPCase activity (Fig. 1) is not correlated with the relatively small increase of LSU mRNA.

As expected from previous experience (Oelmüller et al. 1986), impairment of plastidogenesis, caused by an inhibition of intraplastidic protein synthesis through the application of CAP, not only inhibits synthesis of RuBPCase (see Fig. 1) and LSU mRNA (see Fig. 4) but also the appearance of translatable mRNA for SSU precursors (pSSU)

in light and dark (Fig. 5). This confirms the conclusion drawn previously (Oelmüller et al. 1986) that a signal from the plastid ("plastidic factor") is essential for the expression of the nuclear-encoded SSU gene(s). The CAP effect is specific insofar as CAP (20 $\mu g \cdot cm^{-3}$) does not lead to any detectable effect on growth and development during the period of experimentation (up to 72 h after sowing), and the levels of enzymes not related to plastids are not adversely affected (Oelmüller et al. 1986).

Discussion

The questions as addressed in the *Introduction* can now be discussed.

(1) *Control by phytochrome* of accumulation of RuBPCase and SSU-mRNA is a modulation rather than an induction. The situation in mustard seems to be similar to cucumber and maize where a considerable level of SSU mRNA was detected in dark-grown seedlings (Walden and Leaver 1981; Nelson et al. 1984) while in etiolated pea leaves the amount of SSU mRNA was below detectibility (Bennett et al. 1984; Smith and Ellis 1981). In mustard a rise in the level of the enzyme can be measured 12 h before a response to phytochrome (cFR) can be detected. We conclude that an endogenous control factor turns on RuBPCase synthesis and that the operation of this factor is modulated by phytochrome. This modulation becomes more important as development progresses. From 66 h after sowing onwards, further accumulation of RuBPCase depends entirely on the action of phytochrome (Fig. 1, inset).

(2) *Rates of synthesis,* measured in vivo, of SSU and LSU appear strictly coordinated in light and dark, including the time point of onset of synthesis of both subunits (approx. 33 h after sowing) and the time point (approx. 42 h after sowing) where synthesis becomes responsive to phytochrome (cFR). The data show that the rise of the level of enzyme activity is due to a coordinated synthesis de novo of the subunits.

(3) *The strict coordination* observed at the level of synthesis of subunits is no longer seen on the level of mRNAs. While the measured levels of translatable mRNA for pSSU are compatible with the presumed rate-limiting function in SSU synthesis, the relatively high LSU mRNA levels in dark-grown plants are not reflected in the rate of in-vivo LSU or RuBPCase syntheses.

In particular, the relatively high level of LSU mRNA present in cotyledons before 30 h after sowing is not translated in vivo into a stable protein (compare Figs. 2B and 4). The high level of LSU mRNA in older mustard seedlings (Link 1984) is only doubled in light-grown compared with dark-grown material (see Table 1), in contrast to the results obtained for RuBPCase activity (Fig. 1, inset). Moreover, the SSU mRNA and RuBPCase levels in the mustard cotyledons were found to be potentiated by light pulses operating through phytochrome (Oelmüller et al. 1986; Brüning et al. 1975), whereas the LSU mRNA in the same plant is not significantly affected by light pulses (Link 1982).

On the other hand, the rise of the rate of synthesis 30 h after sowing and the time point of onset of response to phytochrome (cFR) are clearly expressed at the level of LSU mRNA. It seems that a considerable part of the LSU mRNA as detected by dot-blot hybridisation is not involved in LSU synthesis. This inert level seems to be represented by the "base line" in Fig. 4a (□ ■).

(4) *Gene expression* is totally abolished in the case of the SSU if intraplastidic translation and consequently plastidogenesis is inhibited by the application of CAP from the time of sowing onwards, i.e. under conditions where accumulation of LSU mRNA in dark- and cFR-grown plants is largely inhibited. This supports a previous conclusion (Oelmüller et al. 1986) that a "plastidic factor" whose production crucially depends on unimpaired plastid development up to 48 h after sowing is an indispensable prerequisite for expression of the SSU gene(s). However, the inhibition of LSU accumulation as such is not the reason for the failure of SSU (Schmidt and Mishkind 1983) and SSU mRNA to appear (Oelmüller et al. 1986).

Regarding the control mechanism involved in the regulation of the appearance of SSU mRNA, it is obvious that the expression of the SSU gene(s) is turned on by some endogenous control factor (EF) approx. 12 h prior to the onset of responsiveness to phytochrome (Pfr). However, Pfr dominates more and more as development proceeds, and from 66 h after sowing onwards an increase in enzyme level requires active phytochrome. Between 42 and 66 h after sowing (see Fig. 1), EF and Pfr exert their control function simultaneously, presumably in a multiplicative manner, as described previously for the appearance of NADP-GPD (Oelmüller and Mohr 1984). Moreover, it was shown previously (Oelmüller et al. 1986), and confirmed here by the application of CAP, that in light- as well as in dark-grown plants the integrity of the plastid (i.e. an unimpaired state) is essen-

tial for the expression of nuclear genes such as SSU whose protein products are plastid-bound. The present data confirm that a "plastidic factor" is required to allow the appearance of translatable mRNA for SSU. All these postulated factors must be considered in molecular studies which attempt to fully analyze the regulation of RuBPCase gene expression (e.g. Nagy et al. 1985; Dean et al. 1985; Timko et al. 1985).

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