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Translational Regulation of Light-Induced Ribulose 1,5-Bisphosphate Carboxylase Gene Expression in Amaranth

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The regulation of the genes encoding the large and small subunits of ribulose 1,5-bisphosphate carboxylase was examined in amaranth cotyledons in response to changes in illumination. When dark-grown cotyledons were transferred into light, synthesis of the large- and small-subunit polypeptides was initiated very rapidly, before any increase in the levels of their corresponding mRNAs. Similarly, when light-grown cotyledons were transferred to total darkness, synthesis of the large- and small-subunit proteins was rapidly depressed without changes in mRNA levels for either subunit. In vitro translation or in vivo pulse-chase experiments indicated that these apparent changes in protein synthesis were not due to alterations in the functionality of the mRNAs or to protein turnover, respectively. These results, in combination with our previous studies, suggest that the expression of ribulose 1,5-bisphosphate carboxylase genes can be adjusted rapidly at the translational level and over a longer period through changes in mRNA accumulation.

Photomorphogenesis, the phenomenon of growth and development in response to light, provides a valuable experimental system in which to study the control of gene expression by environmental stimuli. In higher plants the development of photosynthetically active chloroplasts is light dependent. Dark-grown plants lack thylakoid organization, chlorophyll, and many of the polypeptides associated with photophosphorylation and CO_2 fixation. Illuminating dark-grown plants causes the accumulation of chlorophyll and induces the development of thylakoid components into photosynthetically functional units. Light also induces changes in the synthesis of a number of nucleus- and plastid-encoded proteins including, in some species, ribulose 1,5-bisphosphate carboxylase (RuBPCase) (1, 32, 33, 36, 37, 43).

RuBPCase is located in the chloroplasts of all higher plants and is the primary enzyme of photosynthetic carbon fixation. This enzyme has a molecular mass of about 550 kilodaltons and consists of eight large subunits (LSUs; 51 to 58 kilodaltons) and eight small subunits (SSUs; 12 to 18 kilodaltons) (29), with the substrate-binding site located on the LSUs (24, 25). The LSU is encoded on the chloroplast genome and is translated on 70S chloroplast ribosomes (9, 13). The SSU is encoded in the nucleus and is translated on free, cytoplasmic ribosomes as a 20-kilodalton precursor (7, 8, 12, 16, 18). The precursor is processed to its final size during transport into the chloroplast, where it assembles with LSUs to form the active holoenzyme.

RuBPCase mRNA or protein accumulation (or both) has been shown to be strongly light dependent in several plant species including pea (2, 10), soybean (6), *Lemna gibba* (38, 44), and *Spirodela oligorhiza* (11, 14). However, light-dependent production of RuBPCase is not universal, since in cucumber (46) and maize (30) similar amounts of the enzyme are produced in dark- and light-grown plants.

Light-induced increases in RuBPCase accumulation have been correlated with similar increases in LSU and SSU mRNA levels in a number of plant species (4, 37, 40, 43, 44). Furthermore, transcriptional runoff experiments with isolated nuclei demonstrated that transcription of SSU genes in pea (15) and *Lemna gibba* (34) is light dependent. These studies suggest that light regulates RuBPCase production primarily at the level of transcription.

We have recently shown that in light- and dark-grown cotyledons of grain amaranth RuBPCase gene expression is regulated not only by the level of mRNA accumulation but also by a posttranscriptional mechanism (5). There have been a few other reports of posttranscriptional control of RuBPCase gene expression in lower plant species such as *Chlamydomonas reinhardtii* (28), *Euglena gracilis* (27), and *Volvox* species (23). Furthermore, this type of regulation has also been documented for another nuclear-encoded chloroplast protein, the chlorophyll *a/b*-binding protein (3, 38, 41, 42).

We report here that in the C_4 dicotyledonous plant *Amaranthus hypochondriacus* rapid and dramatic alterations in the synthesis of the LSU and SSU polypeptides occur in response to changes in illumination. The apparent changes in the rates of synthesis of these polypeptides occur independently of any alterations in LSU or SSU mRNA levels. Moreover, since the stability of these polypeptides does not appear to be affected by changes in illumination, light-mediated translational regulation is implied.

MATERIALS AND METHODS

Plant material and growth conditions. Seeds of *A. hypochondriacus* var. R103 were obtained from the Rodale Organic Gardening and Farming Research Center (21). For light-grown plants, seeds were germinated and plants were grown in a Conviron growth chamber at 24°C with 14 h of illumination per day at an approximate intensity of 160 to 200 microeinsteins m^{-2} s^{-1}, and cotyledons were harvested at the appropriate time points. For the light-induction experiments seeds were germinated and plants were grown in lightproof boxes which were placed in a darkroom. After 8 days these dark-grown seedlings were transferred into the illuminated growth chamber, and cotyledons were harvested at the appropriate time points. For light-to-dark transitions, plants were germinated and grown under normal illumination

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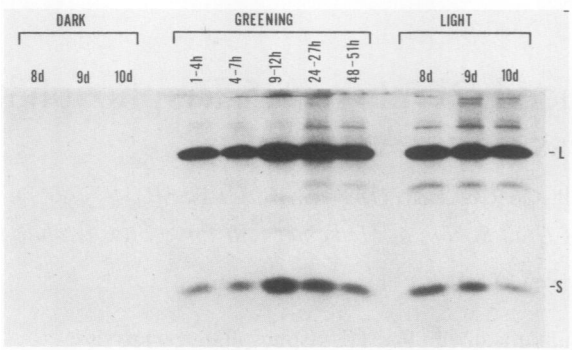

FIG. 1. LSU (L) and SSU (S) synthesis in response to light. Amaranth seedlings were germinated and grown either in complete darkness or under illumination. Eight days (d) after planting, dark-grown seedlings were exposed to light (greening). Excised cotyledons were incubated with [^{35}S]methionine for 3 h at the time periods indicated. LSU and SSU were immunoprecipitated from extracts containing equal amounts of radioactivity incorporated into protein, fractionated by SDS-PAGE, and fluorographed.

conditions for 6 days and then transfered to lightproof boxes in the darkroom. Extreme care was taken during the growth of dark-grown and dark-shifted seedlings to avoid any exposure to light. Cotyledons from dark-grown and dark-shifted seedlings were harvested under a Kodak no. 7 green safelight and immediately frozen in liquid nitrogen or extracted.

Analysis of protein synthesis. Rates of in vivo protein synthesis were determined by radioactively labeling with [^{35}S]methionine. The procedures for labeling, extraction of proteins, immunoprecipitation of LSU and SSU polypeptides, and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were described previously (5).

Pulse-chase experiments to determine the stability of LSU and SSU polypeptides were carried out with 6-day-old cotyledons. After a 2-h pulse with [^{35}S]methionine in a 200-μl solution, the cotyledons were removed from the labeling solution, washed three times with 10 mM methionine, and further incubated in a 0.5-ml solution of 10 mM methionine. At various times into the chase, radioactively labeled proteins from two cotyledons were extracted, and LSU and SSU were immunoprecipitated and analyzed by SDS-PAGE. Light-grown cotyledons were pulsed with 100 μCi of [^{35}S]methionine and chased in the light. In dark-grown cotyledons LSU and SSU synthesis is drastically reduced (5). Thus, to determine the stability of these polypeptides with pulse-chase, 10 times more radioactive label was utilized in the pulse. In light-to-dark transitions the pulse of [^{35}S]methionine and subsequent chase were carried out as described in the legend to Fig. 7.

The effect of the photophosphorylation inhibitors 3-(3,4 dichlorophenyl)dimethylurea (DCMU; Sigma Chemical Co.) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma) on protein synthesis was determined by preincubating for 10 min the excised cotyledons with 5 and 25 μM of each inhibitor, respectively, followed by a 2-h pulse of [^{35}S]methionine in the presence of the inhibitors. Radioactively labeled proteins were extracted and analyzed by SDS-PAGE. The effectiveness of DCMU and CCCP in inhibiting photophosphorylation was established by determining the rate of $^{32}P_i$ incorporation into ATP in treated and untreated cotyledons.

Analysis of mRNA. Total RNA was isolated from cotyledons as previously described (5). RNA was fractionated on agarose-formaldehyde gels and transferred to nitrocellulose paper for Northern analysis by the method of Maniatis et al. (26). LSU and SSU mRNAs were detected by using ^{32}P-labeled cloned LSU (pAls1) or SSU (pAss1) genes from amaranth as probes (5). In vitro translations of total cellular RNA were done by using cell-free lysates prepared from *Escherichia coli* (47) or wheat germ (Bethesda Research Laboratories, Inc.; 31) with a range of RNA concentrations as previously described (5).

RESULTS

Light-induced expression of LSU and SSU genes is regulated posttranscriptionally. The light-mediated stimulation of RuBPCase synthesis was examined by illuminating dark-grown (etiolated) amaranth cotyledons. As our previous results have shown (5), synthesis of both RuBPCase subunits was dramatically reduced in dark-grown seedlings. Upon illumination of these seedlings, RuBPCase synthesis was induced very rapidly (Fig. 1). LSU and SSU synthesis in the cotyledons increased from nearly undetectable amounts to levels similar to that observed in light-grown seedlings within 4 h postillumination. LSU and SSU synthesis was specifically increased 20-fold or more above general protein synthesis during light induction.

LSU and SSU mRNA levels were determined by Northern blot analysis (Fig. 2) with ^{32}P-labeled cloned LSU (pAls1) or SSU (pAss1) genes from amaranth as probes (5). Although synthesis of LSU and SSU polypeptides was dramatically enhanced during the first 4 h after illumination, the corresponding mRNA levels remained constant during this period and did not increase until after 5 h postillumination. Therefore, synthesis of the RuBPCase proteins was induced at the posttranscriptional level before any increase in LSU or SSU mRNA accumulation. By 12 h postillumination the amount of mRNA for both subunits had increased 10-fold, and by 24 h the mRNAs had reached levels normally observed in light-grown seedlings. During this period, when

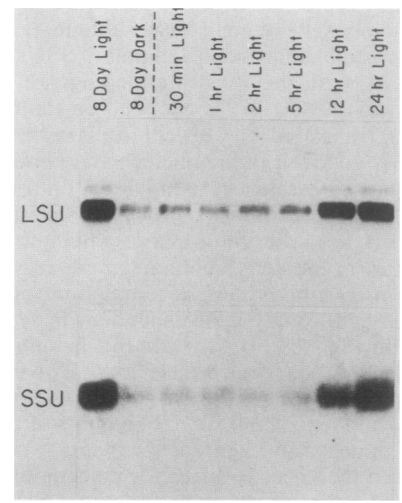

FIG. 2. LSU and SSU mRNA accumulation in response to light. Total RNA was extracted from 8-day-old dark- and light-grown cotyledons and from dark-grown cotyledons exposed to light for the time periods indicated. Samples (5 μg per lane) of total RNA were fractionated on an agarose-formaldehyde gel, transferred to nitrocellulose, and probed with ^{32}P-labeled pAls1 and pAss1.

the mRNA levels increased substantially, the synthesis of both subunits increased only two- to threefold.

To determine whether the induction of RuBPCase synthesis by light involved an alteration in the functionality of the LSU or SSU mRNAs, total RNAs were translated in vitro with cell-free translation systems derived from *E. coli* (for the LSU) and wheat germ (for the SSU). The subunit proteins were immunoprecipitated from the reactions with anti-LSU or -SSU serum and examined by SDS-PAGE. Similar amounts of LSU were produced in vitro by RNA isolated from 8-day-old dark-grown cotyledons and from dark-grown cotyledons after 4 h of illumination (Fig. 3). Similarly, SSU mRNA isolated from 8-day-old dark-grown cotyledons was as translatable in vitro as that isolated from the cotyledons 4 h postillumination (Fig. 3). Since the dark-grown and postillumination mRNAs were equally translatable in vitro, it is unlikely that the posttranscriptional stimulation of RuBPCase synthesis resulted from alterations in LSU or SSU mRNA structure or functionality.

Depression of LSU and SSU synthesis occurs without changes in mRNA during light-to-dark transitions. The effects of light on RuBPCase gene expression were further investigated by transferring light-grown seedlings into darkness. During this light-to-dark transition LSU and SSU protein synthesis and mRNA accumulation were examined.

Upon transfer to darkness, total protein synthesis was only moderately depressed. Total protein synthesis in the cotyledons was reduced approximately twofold by 2 h after transfer and approximately fourfold by 6 h after transfer. Although the synthesis of the majority of the polypeptides was reduced, synthesis of some remained constant, and the production of at least one actually increased (Fig. 4). In contrast, synthesis of both the LSU and SSU declined approximately 20-fold by 2 h and >50-fold by 6 h after the light-to-dark transition (Fig. 4).

The dramatic reductions in LSU and SSU synthesis were not accompanied by changes in the levels of the corresponding mRNAs for up to 6 h after transfer to darkness (Fig. 5).

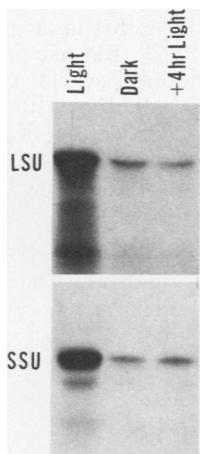

FIG. 3. In vitro translation of RNA from seedlings transferred from the dark to the light. Total RNA was extracted from 8-day-old dark- and light-grown cotyledons as well as from dark-grown cotyledons transferred into the light for 4 h. RNA preparations were used to program cell-free translation systems derived from *E. coli* and wheat germ to test the functionality of mRNAs encoding the RuBPCase LSU and SSU, respectively. LSU and the precursor of SSU were identified among the translation products by immunoprecipitation and SDS-PAGE of equal amounts of trichloroacetic acid-precipitable radioactivity.

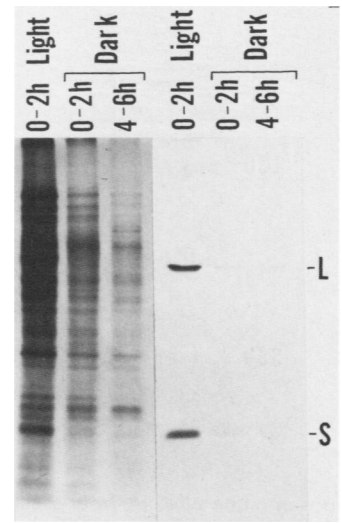

FIG. 4. Total protein synthesis and synthesis of LSU (L) and SSU (S) in response to a light-to-dark transition. Amaranth seedlings were germinated and grown under normal illumination for 6 days and then transferred to total darkness. The seedlings were incubated for 2 h with [^{35}S]methionine at the time periods indicated. To determine changes in total protein synthesis (first three lanes), equal amounts of total protein were fractionated by SDS-PAGE and fluorographed. To determine changes in LSU and SSU synthesis (second three lanes), equal amounts of incorporated radioactivity were immunoprecipitated with anti-LSU and -SSU sera, fractionated by SDS-PAGE, and fluorographed.

In vitro translation of RNA extracted from plants transferred to darkness showed that the levels of translatable mRNA for both LSU and SSU were unaffected by the transition (Fig. 6). Therefore, no changes in mRNA levels of functionality occurred during this time period.

These results show that RuBPCase synthesis was rapidly depressed when light-grown seedlings were transferred to darkness. This shutdown of synthesis occurred posttranscriptionally, without any detectable changes in the LSU or SSU mRNAs.

Translational control of LSU and SSU synthesis. One pos-

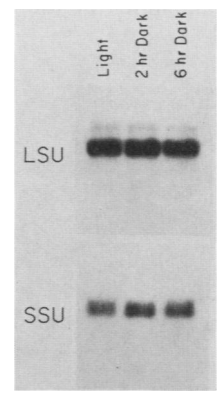

FIG. 5. LSU and SSU mRNA accumulation in response to a light-to-dark transition. Total RNA was extracted from 6-day-old, light-grown cotyledons and from cotyledons transferred to darkness for the time periods indicated. Samples (5 μg per lane) of total RNA were fractionated on an agarose-formaldehyde gel, transferred to nitrocellulose, and probed with ^{32}P-labeled pAls1 and pAss1.

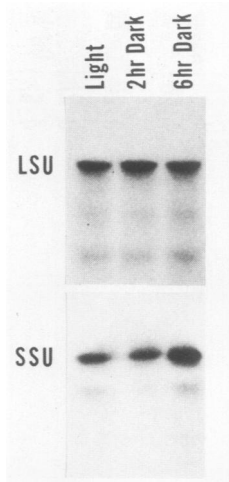

FIG. 6. In vitro translation of RNA from seedlings transferred from the light to the dark. Total RNA was extracted from 6-day-old light-grown cotyledons as well as from cotyledons transferred into darkness for 2 or 6 h. The functionality of mRNAs encoding the LSU or SSU of RuBPCase was tested by in vitro translation in *E. coli* or wheat germ cell-free systems, respectively. Translation products were analyzed by immunoprecipitation and SDS-PAGE of equal amounts of trichloroacetic acid-precipitable radioactivity.

sible mechanism for the observed posttranscriptional block to the synthesis of LSU and SSU polypeptides is their more rapid turnover in the absence of light. To test this possibility we examined the relative stability of newly synthesized polypeptides by pulse-labeling with [^{35}S]methionine and chasing for up to 8 h with excess unlabeled methionine. The effectiveness of the case was confirmed by the cessation of the incorporation of radioactivity into proteins during the chase period. The LSU and SSU polypeptides were immunoprecipitated from crude protein extracts and analyzed by SDS-PAGE (Fig. 7). In light-grown amaranth cotyledons, turnover of LSU and SSU could not be detected during an 8-h or even a 24-h (data not shown) chase, consistent with earlier studies which indicated that these proteins are very stable, with half-lives on the order of days (20, 35). More importantly, LSU and SSU exhibited similar stabilities in light- and dark-grown cotyledons. Furthermore, when the pulse-chase experiments were carried out during a light-to-dark transition, the stabilities of the RuBPCase subunits were unaltered.

These experiments demonstrate that the posttranscriptional block to the synthesis of LSU and SSU polypeptides in dark-grown seedlings and during a light-to-dark transition was not due to enhanced turnover of these polypeptides. Also, since there was no change in the functionality of the corresponding mRNAs, we conclude that the mechanism blocking synthesis of the RuBPCase subunits operates at the translational level.

Cessation of synthesis is not directly due to energy depletion. During a light-to-dark transition, synthesis of RuBPCase subunits was rapidly and specifically reduced. Upon illumination of these seedlings, translation was rapidly resumed (data not shown). Since the major source of cellular energy in plants is derived from photophosphorylation, it is conceivable that this reduction in the synthesis of the RuBPCase subunits could be due to deprivation of energy. Indeed, ATP levels in chloroplasts and cytoplasm of mesophyll cells are reduced in the dark (39).

To test the possibility that LSU and SSU synthesis may be directly regulated by cellular ATP levels during a light-to-dark transition, cotyledons were preincubated with an inhibitor of photosynthetic electron transport, DCMU, or an uncoupler of photophosphorylation, CCCP, before the determination of protein synthesis. DCMU (Fig. 8, lanes b and e) and CCCP (Fig. 8, lanes c and f) had no apparent effect on total protein synthesis. In particular, these inhibitors did not affect LSU and SSU synthesis, even though their synthesis was dramatically reduced in the dark (Fig. 8, lane d). The effectiveness of DCMU and CCCP in inhibiting photophosphorylation was established by determining the rate of $^{32}P_i$ incorporation into ATP in treated and untreated cotyledons. DCMU and CCCP reduced $^{32}P_i$ incorporation into ATP to the same low levels observed in untreated cotyledons after a light-to-dark transition (results not shown). These experiments suggest that the depression of LSU and SSU synthesis during a light-to-dark transition was not due to depletion of cellular ATP in the dark.

DISCUSSION

We have already shown that in amaranth cotyledons the light-mediated control of RuBPCase production is complex (5). This is due to the presence of at least two levels of regulation, transcriptional and posttranscriptional, which determine the final accumulation of the LSU and SSU polypeptides. In this report we extend the previous studies by providing evidence that light-mediated, posttranscrip-

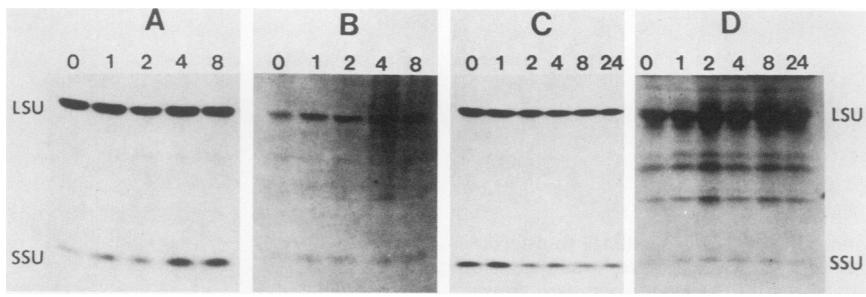

FIG. 7. Stability of LSU and SSU polypeptides in amaranth cotyledons under different illumination conditions. Ten 6-day-old cotyledons were excised and pulse-labeled for 2 h with [^{35}S]methionine. Cotyledons were then washed with 10 mM nonradioactive methionine and further incubated in this solution. At the indicated times (hours) after the pulse, labeled proteins were extracted from two cotyledons, analyzed by immunoprecipitation with anti-LSU and -SSU sera, and fractionated by SDS-PAGE. A, Cotyledons grown in the light were pulsed (100 μCi of [^{35}S]methionine) and chased in the light. B, Cotyledons grown in the light were both pulsed (1 mCi of [^{35}S]methionine) and chased immediately after a light-to-dark transition. C, Cotyledons grown in the light were pulsed (100 μCi of [^{35}S]methionine) in the light and chased in the dark. D, Cotyledons grown in complete darkness were pulsed (2 mCi of [^{35}S]methionine) and chased in the dark.

tional regulation of RuBPCase production occurs at the level of translation.

Both our previous and present data indicate that alterations in the synthesis of the RuBPCase proteins can occur without corresponding changes in LSU or SSU mRNA levels or changes in their translatability in vitro. When 8-day-old dark-grown seedlings were exposed to light, synthesis of both the LSU and SSU polypeptides was greatly increased during the first 4 h of illumination. However, their corresponding mRNAs did not change until after 5 h of illumination, well after RuBPCase synthesis was dramatically enhanced. When cotyledons grown under normal illumination conditions were transferred to total darkness, synthesis of the LSU and SSU polypeptides was rapidly depressed. This shutdown of RuBPCase synthesis occurred in the absence of any changes in the LSU or SSU mRNA levels or functionality. When these seedlings were transferred back into light, RuBPCase synthesis was reinitiated within 2 h postillumination, again without any changes in the mRNAs. These results suggest that amaranth seedlings can respond rapidly to changes in illumination at the posttranscriptional level without any corresponding changes in mRNA synthesis, processing, or breakdown.

Additional evidence that the synthesis of both the LSU and SSU polypeptides is not tightly coupled to the levels of their respective mRNAs was provided by the light induction experiments. The LSU and SSU mRNA levels were 20-fold lower in 8-day-old dark- versus light-grown seedlings and did not change during the first 5 h of illumination. Despite these large differences in mRNA levels, LSU and SSU protein synthesis during the first few hours of illumination was only slightly less (two- to threefold) than that observed in the light-grown seedlings. In addition, whereas the mRNA levels increased substantially at 12 h postillumination, LSU and SSU synthesis increased only slightly at this time. These results suggest that as the mRNAs became more abundant,

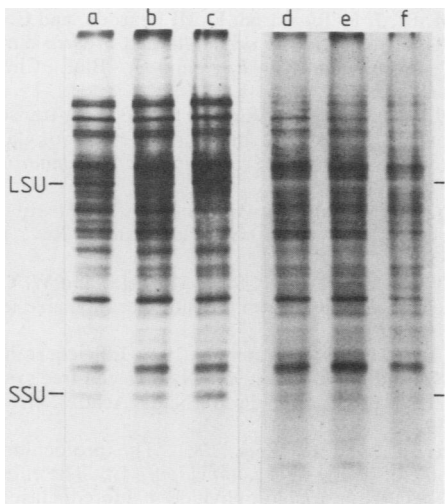

FIG. 8. Effect of the photophosphorylation inhibitors DCMU and CCCP on LSU and SSU synthesis in amaranth cotyledons. Excised amaranth cotyledons were pretreated with 10 min with H_2O (lanes a and d), 5 μM DCMU (lanes b and e), or 25 μM CCCP (lanes c and f) before labeling for 2 h with 100 μCi of [^{35}S]methionine in the presence of the inhibitors. The pretreatment and subsequent labeling were carried out in the light (lanes a, b, and c) or immediately after a light-to-dark transition (lanes d, e, and f). Radioactively labeled proteins were extracted from equal numbers of cotyledons and analyzed by SDS-PAGE.

the efficiency of their utilization progressively decreased. The reason for this apparent underutilization of RuBPCase mRNAs in the light (or their more efficient use shortly after the transfer from dark to light) is not yet clear.

The posttranscriptional block in dark-grown or dark-shifted cotyledons might be due to a more rapid turnover of the newly synthesized proteins in the absence of light. However, our pulse-chase experiments failed to detect any difference in stability of these polypeptides under the different light conditions. Unfortunately, we cannot rule out the possibility that there may exist two subpopulations of one or both subunits: one that is stable and detected in our experiments and another that is rapidly turned over and, due to the length of the pulse labeling, undetected. Nonetheless, taken together our results suggest that alterations in the expression of RuBPCase genes in response to changes in illumination were not due to alterations in the LSU or SSU mRNAs or to differences in protein turnover. Rather, the amaranth seedlings appear to rapidly respond to changes in illumination by altering RuBPCase gene expression at the translational level.

The light-induced expression described here may be specific to a subset of genes including those encoding the LSU and SSU polypeptides. When dark-grown seedlings were transferred to light, LSU and SSU synthesis was greatly enhanced relative to total protein synthesis. In addition, during a light-to-dark transition RuBPCase synthesis was reduced much more dramatically than total protein synthesis. However, since the chloroplast, in contrast to the nucleus, encodes relatively few polypeptides, and these are not readily discernable by SDS-PAGE, it was difficult to determine whether translation by 70S chloroplastic ribosomes was more drastically reduced than total protein synthesis in dark-grown cotyledons. If this were the case, then the translational block to LSU synthesis could be due to a more general cessation of chloroplastic protein synthesis.

To investigate the question of specificity with respect to light regulation of LSU synthesis, several approaches were employed. When the production of another chloroplast-encoded protein, the β-subunit of ATPase, was analyzed, it was also found to be light regulated (data not shown). In contrast, preliminary studies with drugs which specifically inhibit either chloroplastic (chloramphenicol) or cytoplasmic (cycloheximide) protein synthesis suggest that significant protein synthesis continued in dark-grown and in dark-shifted chloroplasts, even though the production of two known polypeptides was essentially turned off. Surprisingly, the results from these experiments indicated that in dark-grown seedlings chloroplastic protein synthesis in fact constituted a higher proportion of total protein synthesis than in light-grown seedlings (as determined by trichloroacetic acid-precipitable radioactivity). Other reports have also shown that several chloroplastic proteins are synthesized in immature chloroplasts in dark-grown plants (17, 22, 45). Although these latter results lend support for specific regulation by light of the synthesis of a subclass of chloroplast-encoded polypeptides, further investigation is required to rigorously establish this.

The results presented here and in a previous report (5) demonstrate that amaranth seedlings can respond to changes in illumination by altering gene expression both transcriptionally and translationally. For the LSU and SSU genes, rapid responses to changes in the environment appear to be accomplished at the translational level, whereas long-term changes in gene expression are regulated at the transcriptional level. Although there are many examples of transcrip-

tional regulation of gene expression in both plants and animals, relatively few instances of regulation at the translational level have been reported (19). This work and recent studies with *Spirodella* species (14) and *Volvox* species (23) provide some of the first evidence for the importance of translational regulation of gene expression in plants.

ACKNOWLEDGMENTS

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