Control of *psbA* gene expression: in mature *Spirodela* chloroplasts light regulation of 32-kd protein synthesis is independent of transcript level

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Spirodela oligorrhiza plants were used to study direct effects of light on plastid gene expression uncoupled from sequential chloroplast-developmental processes. Specific transcript levels were analysed using chloroplast, gene-fragment probes. Protein synthesis *in vivo* and *in vitro* was also measured. In tissue with mature chloroplasts, light/dark regimes had no substantial effect on transcript levels of the *psbA* gene coding for the 32-kd protein. However, under the same conditions, synthesis of the protein itself was dramatically affected. We conclude that in mature chloroplasts synthesis of 32-kd protein is regulated mainly at a translational level. Transcript levels of the *rbcL* gene, coding for the large subunit of ribulosebiphosphate carboxylase-oxygenase, were somewhat sensitive to light/dark effects, although not to the same degree as was synthesis of the protein. We conclude that translational events and transcript levels are involved in regulating synthesis of this polypeptide. Carbon deprivation in the dark reduced *psbA* and *rbcL* transcript levels appreciably below those found in non-starved, dark-grown tissue. This suggests that starvation and dark effects must be experimentally separated from each other for valid conclusions to be drawn about light/dark regulation of chloroplast gene expression.

Key words: messenger RNA/ribulosebiphosphate carboxylase-oxygenase/*Spirodela oligorrhiza*/translational control

Introduction

Dramatic changes occur in plastids upon transfer from steady-state dark growth to light. These alterations include accumulation of chlorophyll and thylakoid membranes, accumulation of mRNAs, and a marked enhancement of chloroplast protein synthesis. Some of the light-stimulated proteins, such as the light-harvesting chlorophyll a/b apoprotein (LHCP) and the small subunit of ribulosebiphosphate carboxylase-oxygenase (SS), are nuclear coded. Others, such as the 32-kd photosystem II polypeptide and the large subunit of ribulosebiphosphate carboxylase-oxygenase (LS), are chloroplast coded. The levels of the mRNAs for the two nuclear-coded chloroplast proteins were found to be reversibly regulated by light. In *Lemna* (Tobin, 1978; Tobin and Suttie, 1980), it was shown that transfer of mature green tissue to the dark caused a rapid and specific decrease in the level of these mRNAs, while re-exposure to the light resulted in a rapid recovery of the mRNA levels. A similar, reversible light/dark change in mRNA level has been elegantly demonstrated for pea SS gene in transformed petunia cells (Broglie *et al.*, 1984).

The effect of light on chloroplast mRNA levels has been extensively studied during plant or plastid maturation. Under these developmental conditions, marked increases were observed in transcript levels for the *psbA* gene, coding for the 32-kd protein (Bedbrook *et al.*, 1978; Reisfeld *et al.*, 1978) and the *rbcL* gene, coding for the LS polypeptide (Smith and Ellis, 1981; Walden and Leaver, 1981; Poulsen, 1984; Crossland *et al.*, 1984). Based on these results it has become generally accepted (Smith and Ellis, 1981; Edelman, 1981; Link, 1982; Thompson *et al.*, 1983) that the transcript levels for these chloroplast genes are light regulated. However, this supposition has never been directly tested, as was done for the nuclear-coded chloroplast transcripts, where mRNA levels were shown to be reversibly affected by light/dark regimes. Such experiments would be valuable in distinguishing between a role for light as a general effector of plant developmental processes, or as a specific regulator of chloroplast transcript levels.

The aquatic higher plant *Spirodela oligorrhiza* can grow as a phototroph in the light and as a heterotroph in the dark. When placed in the dark, mature green plants retain their chlorophyll and photosynthetic membrane ultrastructure for at least 3 months while remaining fully viable (Porath, 1979). Using such plants, Mattoo *et al.* (1984) have recently suggested that the rate of synthesis of the 32-kd polypeptide is dependent on photophosphorylation within the chloroplast. However, there was no attempt to determine whether transcript levels and/or translation were affected by limiting light. In the present study we analyse protein synthesis and transcript levels for the *psbA* gene in light- and dark-grown, green *Spirodela* plants. Contrary to prevailing assumptions, and in contrast to what was shown for nuclear-coded genes, we find that the level of this major chloroplast-coded transcript is stable in the dark under conditions where protein synthesis is curtailed. We conclude that expression of the 32-kd protein is, indeed, light regulated; however, this regulation mainly occurs at a translational stage.

Results

The effect of darkness on specific transcript levels and in vivo protein synthesis

Spirodela were grown under continuous light on minimal medium supplemented with 1% sucrose and then transferred to the dark for various periods. Plants were either incubated with [³⁵S]methionine, or collected for RNA isolation. The rate of uptake of [³⁵S]methionine into light-grown and dark-grown plants was similar (Figure 1A), although total protein synthesis decreased with increasing culture time in the dark (Figure 1B). Gel fractionation showed that the 32-kd and LS chloroplast polypeptides were among those proteins whose synthesis declined rapidly (Figure 1C). In contrast, the levels of the transcripts coding for these same polypeptides were far less affected by the absence of light. Figure 2A and B shows the results of hybridising *psbA* and *rbcL* gene probes to total

Fig. 1. Effects of darkness on *in vivo* protein synthesis. *Spirodela* plants were grown on heterotrophic media in the light and transferred to darkness for various periods. (A) Rate of [^{35}S]methionine uptake. Light-grown control plants (●) and plants cultured in darkness for 16 h (▲) and 500 h (■) were incubated for 0, 0.5, 1, 2 and 4 h with [^{35}S]methionine as described in Materials and methods. At the times indicated, 1% of the culture medium was counted in a liquid scintillation spectrometer (100% on the abscissa represents 25 000 c.p.m.). (B) [^{35}S]methionine incorporation into the trichloroacetic acid insoluble fraction. Plants were treated as in (A) and collected and homogenised as described in Materials and methods. Aliquots of the homogenate were precipitated with trichloroacetic acid on 3 MM paper disks according to Roberts and Patterson (1973). The radioactivity at each time point represents the counts in 4 μg protein. (C) Protein synthesis patterns following dark growth. Light-grown plants were transferred to darkness for 0 h (light-grown control), 16 h and 500 h. Plants were labelled with [^{35}S]methionine and their proteins extracted. Whole cell homogenate, containing 15 μg of protein, was loaded on each lane of a SDS 10–20% polyacrylamide gel. Electrophoresis and fluorography were as described (Hoffman-Falk *et al.*, 1982). The locations of the LS, 32-kd, LHCP and SS polypeptides are indicated.

RNA gel blots. Only after 500 h of darkness was an appreciable decrease in the level of either transcript readily discernible on the Northern blots.

The data from several experiments were quantified and are summarised in Figure 3. After 16 h of darkness, the level of *Spirodela* RNA hybridising to the *psbA* gene probe was virtually unchanged from that found in light-grown samples; however, 32-kd protein synthesis was reduced to 15% of that in light controls. Even after 500 h of darkness, the level of 32-kd-specific RNA in the mature green plants remained at 60% of that in the light-grown samples, while 32-kd protein synthesis was reduced to 2% of the light control values. As can be seen in the lower frame of Figure 3, the disparities between *rbcL* transcript levels and the decay in LS protein synthesis in the dark were not as dramatic as those detailed above for the 32-kd protein. Nonetheless, the ratio of *rbcL*-transcript-level to LS protein synthesis increased 2.5- and 3.3-fold over light controls at 16 h and 500 h darkness, respectively.

Aliquots of these same RNA samples were translated in a rabbit reticulocyte cell-free system (Figure 4). The complex nature of the *in vitro* translation reaction precludes quantitative analysis of the products. Nevertheless, from the results shown it is clear that the *psbA* and *rbcL* transcripts are active *in vitro* and produce full-size protein products; i.e., the LS protein and the 33.5-kd precursor to the 32-kd protein. The precursor for the SS protein, pSS, is also a major *in vitro* translation product of RNA from light-grown plants (0 h sample). However, the level of this nuclear mRNA decreases rapidly in the dark as was previously shown in *Lemna* (Tobin and Suttie, 1980).

Effects of starvation on specific transcript levels

In *Spirodela*, dark effects can be studied separately from starvation effects by culturing plants in darkness with or without a carbon source. In the experiment shown in Figure 5, plants were cultured in continuous light in the presence or absence of sucrose in the medium, and then transferred to darkness. After various periods, green plants were collected and the transcript levels of the *psbA* and *rbcL* genes analysed by probe hybridisation. Carbon deprivation in the dark (i.e., starvation) enhanced the decline of the transcript levels of both genes compared with their respective transcript levels in plants cultured under heterotrophic dark conditions.

Discussion

In this study we measured transcript levels and protein synthesis for the 32-kd and LS proteins in the light and after various periods of growth in darkness. Our purpose was to determine whether or not the translation of these two major chloroplast products was coupled to their transcript levels. We were not concerned with synthesis or turnover of transcripts and, thus, have no information on how a particular mRNA level is achieved or maintained *in vivo*. As a common basis for comparison, we analysed green light-grown plants and green plants which persist for several months in the dark, in a fully viable state, with largely unaltered thylakoid structure (Porath, 1979). These latter plants rapidly synthesise the 32-kd polypeptide upon transfer back to light (Mattoo *et al.*, 1984).

Mattoo *et al.* (1984) found that *in vivo* synthesis of the 32-kd polypeptide in *Spirodela* is limited by light-dependent

Fig. 2. Effects of darkness on transcript levels. *Spirodela* plants transferred to darkness for various periods were collected into liquid air and their RNA extracted, fractionated, blotted and hybridised to specific gene probes. Light-grown control plants (lanes 0) and plants placed in darkness for 16 h or 500 h (lanes 16 and 500, respectively) were assayed. Only mature green plants were taken for analysis in the 500 h sample. (A) Hybridisation of fractionated, whole cell RNA to a DNA probe specific for the *psbA* transcript (2 day exposure). Nick-translation of probe and hybridisations were as described (Fluhr and Edelman, 1981). The marker lane (M) contains *Bgl*I and *Eco*RI + *Sal*I digestion fragments of pBR322. The DNA fragments were glyoxylated and fractionated as for RNA samples. Lanes on the 1.5% agarose gel were loaded with 2.5 or 5.0 µg RNA as indicated. (B) Hybridisation of fractionated whole cell RNA to a DNA probe specific for the *rbcL* transcript (6 day exposure). Other details as in (A). Arrow indicates the major LS transcript (approximate size, 1800 bases).

phosphorylation. It is obvious from our results that exclusion of light hardly decreased the *in vivo* transcript levels for this protein. We conclude that in mature green plants expression of the 32-kd protein gene is mainly controlled at the translational level. Only a massive drop in the overall cellular RNA level upon transfer from light to dark and, conversely, a massive increase in total RNA upon transfer back to light, would make this conclusion inadequate. Such events seem unlikely, since green plants grown under any of the light regimes described yielded similar quantities of total extracted RNA per gram fresh weight. For 12 samples examined, a mean value of 111 µg (SE = 8) was obtained. Grouping the dark-grown samples gave a value of 113 µg (SE = 11, $n = 9$) while the light-grown samples gave a value of 105 µg (SE = 6, $n = 3$). Heterotrophically grown samples gave 106 µg (SE = 13, $n = 7$), and carbon deprived samples gave 118 µg (SE = 9, $n = 5$). The possibility that the protein synthesis patterns obtained in Figure 1C can be explained by rapid turnover of the 32-kd or LS polypeptides in the dark can be ruled out as well. Turnover of both proteins was found to be minimal in the dark in *Spirodela* (Mattoo *et al.*, 1984; and unpublished). We conclude that light has little specific regulatory effect in determining the level of mRNA coding for the 32-kd

Fig. 3. Relative changes in transcript levels and *in vivo* protein synthesis as a percent of light control. Plants were treated as described in Figures 1 and 2. Hybridisation levels and protein synthesis were determined by scanning X-ray autoradiograms of Northern blots or fluorograms of protein gels. Percent values for transcript levels represent the average of at least four hybridisation experiments, and those for protein synthesis, the average of two labelling experiments, each analysed in two different gel runs. All microdensitometer readings were normalised to the corresponding values for light-grown control plants (lanes 0) which were set at 100%. Lanes 16 and 500 represent plants transferred to the dark for 16 h and 500 h, respectively. The bar at the top of each histogram represents the standard error. The upper and lower frames show results for 32 kDa and LS, respectively.

polypeptide in mature green *Spirodela* plants. Thus, observations of increasing *psbA* transcript levels during the process of greening (Bedbrook *et al.*, 1978; Reisfeld *et al.*, 1978; Hallick *et al.*, 1982), and during phytochrome-induced chloroplast maturation (Link, 1982; Sasaki *et al.*, 1983; Thompson *et al.*, 1983), may relate more to light-regulated developmental events than to a specific light effect on transcript levels.

The data summarised in Figure 3 shows that the LS transcript level declined moderately in the dark. This decline can be abolished by a 2 min pulse of light given intermittently every 2 h during dark growth (M.Devic *et al.*, in preparation). We note, also, that green *Spirodela* plants do not immediately regain the ability to synthesise the LS polypeptide in the light following several weeks of dark growth (Mattoo *et al.*, 1984). Thus, light/dark regimes do appear to have a significant effect on *rbcL* transcript levels, while at the same time revealing a definite increase in the ratio of *rbcL* transcript level to LS protein synthesis during dark growth. Apparently, LS protein synthesis is regulated by both transcript level and translational events. In this regard, Fromm (1983), Poulsen (1984) and Crossland *et al.* (1984) have indicated the presence of two LS transcripts. The level

Fig. 4. *In vitro* translation as a function of growth in the dark. Plants were grown and treated as described in the legend to Figure 2. Total RNA samples (1, 2 and 4 μ g) were translated in a reticulocyte cell-free system (Pelham and Jackson, 1976). Lane 0 shows translation activity in the reticulocyte lysate fraction without the addition of plant RNA. Arrows indicate the locations of the LS, 33.5-kD (precursor of 32-kD), and pSS (precursor of SS) translation products.

Fig. 5. Effects of carbon deprivation on transcript levels. Plants were grown under continuous light, either on mineral (– sucrose) or heterotrophic (+ sucrose) media, and then transferred to darkness for various periods. Whole cell RNA was isolated, fractionated, blotted and hybridised to DNA probes specific for the *psbA* and *rbcL* transcripts as described in Materials and methods. Autoradiograms of Northern blots were quantified by scanning in a DU-8 Beckman spectrophotometer. The value for each time point represents the average of at least four hybridisation experiments. Transcript levels in plants grown for various periods in the dark are presented as the percentage of the transcript level in light-grown control plants (0 h). Bars represent standard error values.

of the smaller, less abundant transcript is less affected by starvation and light/dark regimes.

Carbon deprivation intensified the declines in levels of both *psbA* and the major, 1800 base *rbcL* transcript in the dark. It is an open question whether this starvation effect is simply an amplification of the dark effect or whether a unique mechanism is involved in the control of chloroplast gene transcript levels under this condition. Based on our results with *Spirodela* we suggest that these two factors may interact, making it

difficult to conclude specifically about the effect of darkness *per se*, unless the two parameters are studied separately, as was done here.

Materials and methods

Plant material and growth conditions

Axenic *S. oligorrhiza* (Kurtz) Heglem was cultured in 250 ml Erlenmeyer flasks containing 100 ml of either mineral medium (half strength Hutner's medium; Posner, 1967) or heterotrophic medium, (mineral medium supplemented with 1% sucrose). Plants were grown at 25°C under fluorescent light (3000 lux), or in the dark in light-proof boxes.

In vivo labelling of proteins

Plants were pre-incubated overnight in 3 ml of fresh medium in 3 cm (diameter) plastic Petri dishes, with half of the surface covered with plants. Prior to radiolabelling, 2.5 ml of medium were removed and 5 μ l (55 μ Ci) of [35 S]-methionine (1400 Ci/mmol, Amersham) were added. After each labelling period, plants were washed several times with cold, sterile water and kept at –70°C (on dry ice) until homogenised. Whole cell homogenate from plants of a single Petri dish was obtained by 20 strokes in an ice-cold, motor-driven (1000 r.p.m.), ground glass-glass homogeniser containing 0.5–1.0 ml of 2.5 mM Tris-glycine, pH 8.5, and 0.15 M NaCl. For the 500 h darkness point, the mature green plants were manually separated from etiolated plants following the radiolabelling and freezing steps but prior to homogenisation. Only the green plants were analysed. Polyacrylamide gel electrophoresis, gel fixation and fluorography were performed as previously described (Hoffman-Falk *et al.*, 1982).

Isolation of RNA

RNA was isolated from whole plants by a modification of the method described by Glisin *et al.* (1974). Plants were collected and kept in liquid air. Frozen plants were ground to a fine powder in a mortar and pestle. A buffer containing 2% Tris-isopropylthiuroniumsulfonic acid (sodium salt), 1% NaCl, 0.05 M Tris-HCl, pH 7.6, and 100 μ M aurin tricarboxylic acid (Sigma) was gradually added up to 5 ml/g fresh plant material. Following centrifugation at 17 000 g for 15 min at 4°C, the supernatant was recovered and 1 g of solid CsCl was added per ml solution. 3.5 ml of this mixture were then layered over 1.2 ml of a solution containing 1.707 g/ml CsCl in 0.05 M Tris-HCl, pH 7.6. Centrifugation was performed for 12 h at 90 000 g at 19°C in an SW-55Ti Beckman rotor. The pellets were resuspended in a minimal volume (0.5 ml/tube) of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, containing 0.1% SDS, and the solutions extracted once with neutralised phenol and once with chloroform:isoamylalcohol (24:1). RNA was precipitated by bringing the solution to 0.2 M with respect to sodium acetate and adding 2.5 vol ethanol. The precipitate was resuspended in sterile distilled water and stored at –70°C. RNA concentrations were determined spectrophotometrically at 260 nm.

Northern blots, hybridisation and in vitro translation

RNA denaturation, fractionation and transfer to nitrocellulose sheets were carried out as described by Thomas (1980) except that vertical 1.5% agarose gels were used. Nick-translation of DNA probes and hybridisation conditions were previously described (Fluhr and Edelman, 1981). Rabbit reticulocyte lysate was prepared and used for *in vitro* translation according to Pelham and Jackson (1976).

Chloroplast gene probes

The gene probe used for the *rbcL* transcript was plasmid pZmc461, which contains a 567-bp internal *Pst*I fragment of the maize *rbcL* gene (McIntosh *et al.*, 1980). The gene probe for the *psbA* transcript was plasmid Sp247(5), which contains an ~750-bp internal *Pst*I fragment of the *Spirodela psbA* gene (Fluhr *et al.*, 1983).

Quantification of protein synthesis and transcript levels

X-ray films were preflashed, autoradiographically or fluorographically exposed, and analysed using a Beckman DU-8 spectrophotometer containing a gel scanning attachment, as detailed by Marder *et al.* (1984).

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