THE EFFECT OF LIGHT EXPOSURE ON THE ACCUMULATION OF RNA TRANSCRIPTS FOR PEA CHLOROPLAST GENES

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ABSTRACT

I am investigating the effects of light on the accumulation of mRNA for the atpF and rbcL chloroplast genes. Light exposure affects the expression of some plant genes. Chloroplasts are genetically semi-autonomous when compared with the rest of the cell and have their own regulatory mechanisms. Using Northern blot analysis we examined the steady state levels of transcripts of atpF and rbcL. We determined that the levels of rbcL-hybridizing transcripts were photoregulated, as has been reported (2). We have not yet gotten good results for the atpF gene transcripts.

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INTRODUCTION

My research involved an investigation of how exposure to light affects the accumulation of RNA messages in pea coding for two chloroplast-encoded proteins: the CF_0 subunit I of the ATP-synthesizing complex (CF_0F_1ATPase) of the thylakoid membrane (1) and the large subunit of ribulose bisphosphate carboxylase/oxygenase (2). The research consisted of preparation of total RNA extracts from pea leaves exposed to light for various amounts of time, and comparison of the levels of mRNA transcripts of the two chloroplast genes in an attempt to determine whether the genes are photoregulated.

Light exposure has dramatic effects on plant and chloroplast development. When plants are germinated and grown in the dark, they become etiolated. The plant itself is yellow because of the lack of photosynthetically active chlorophyll. Chloroplasts in plants grown under lightless conditions are called etioplasts. The colorless etioplasts are not synthesizing photosynthetic pigments, nor are they synthesizing proteins needed to convert light into chemical energy (3).

When the plant is exposed to light, a series of metabolic and morphological changes occur. The leaves unfold and turn green as chlorophyll precursors are rapidly converted to chlorophyll. Chloroplasts

assemble the thylakoid membrane proteins needed for photosynthesis and the plant can begin to transform light into the chemical energy on which it depends for carrying out its energy-requiring processes. Many of these light-dependent changes are brought about by changes in the expression of chloroplast and nuclear genes. Expression of these genes is controlled at many levels. For the production of photosynthetically competent chloroplasts, photocontrol may be at the level of transcription (DNA-dependent mRNA synthesis), mRNA processing, translation (synthesis of protein directed by mRNA), the transport and processing of proteins, and their assembly into functional complexes (3,4).

The changes in light that induce photomorphogenic changes are not related to the use of light as energy. In etiolated seedlings, the light induced developmental effects occur before the photosynthetic pathway is operational, and the changes of gene expression involve levels of light that are much too low to power photosynthesis (5).

For photoregulation of gene expression to occur, there must be a receptor to absorb the light signal, converting this environmental cue to a molecular change that can be recognized and responded to by the host. There are three known photoreceptors in higher plants. They are protochlorophyllide, one or more blue light-ultra violet receptors, and phytochrome. Protochlorophyllide is an intermediate in the chlorophyll synthesis pathway, and exposure to red light rapidly converts it to

chlorophyllide, allowing chlorophyll to accumulate. The blue light-ultra violet receptors are more important in lower plants. Phytochrome is the best characterized photoreceptor in higher plants. It is responsible for the control of many aspects of a plant's existence, including flowering and the regulation of many nuclear and chloroplast genes (5,6,7). Phytochrome consists of two photointerconvertable forms. The inactive form, called P_r, is converted to the active form, called $P_{fr,}$ by exposure to red light. This conversion is reversed by exposure to far red light. Phytochrome regulation has different effects on the genes it controls, but the characteristic of its presence is the induction of change by red light which is reversed by far red light (3,8).

The actual mechanism of the transduction of light signals and the intervening steps leading to changes in gene expression is a biochemical 'black box'. (4) The final changes in response to light stimuli have been mapped, but the overall mechanism is unknown. Changes in gene expression have usually been studied by attempting to 'characterize the complex nature of a plant's response to light at a molecular level.' (8) Often, the change studied is the abundance of an mRNA transcript for a particular gene, measured by Northern blot analysis. The end result of this procedure is a photograph that shows the sizes and relative abundance of the radioactively-labeled transcripts of the gene being studied. The main disadvantage of Northern analysis is that it only measures steady-state

RNA levels. It cannot distinguish between changes in transcription rate, RNA processing, and degradation, while changes in total RNA levels may reflect regulation by any of these means (2). Studies have shown that some genes are controlled largely by transcription rate (4), or show a greater regulatory dependence on mRNA stability (9), or are regulated at the level of translation (10) or by as protein turnover (8). Some researchers have claimed that transcriptional regulation is of minor importance for many chloroplast genes, stating the effects of light on the state of chloroplast development have not been distinguished from more specific transcriptional effects (2). The transcriptional activities of many chloroplast genes are shown to be relatively constant throughout development and increase simultaneously upon light exposure. This limited transcriptional regulation may indicate that the expression of these plastid genes is under posttranscriptional regulation (11). Regardless of these limitations, analysis of steady state mRNA levels does give some information on the regulatory mechanisms present.

The regulation of chloroplast development is complicated. Chloroplasts are the cell organelles that are responsible for the transduction of light energy into the chemical energy that the plant uses to fuel its metabolic processes. Chloroplasts are metabolically and genetically semi-autonomous when compared to the rest of the cell . They have their own DNA coding for a number of products used by the plastid.

The genes in the plastid DNA code for two major groups of products: proteins involved in photosynthesis, such as subunits of the two photosystems and the ATP synthase complex, and those products that create the machinery of gene expression, such as the genes for rRNAs, tRNAs, and some of the ribosomal proteins (12). Chloroplasts are not totally independent of the rest of the cell. Most of their proteins, including some vital to the photosynthetic pathway, are encoded in the nuclear DNA, synthesized as precursors on cellular ribosomes, and imported into the chloroplast as precursors. The proper development of the chloroplast requires the coordinated, regulated expression of both nuclear and chloroplast genes (13).

Chloroplasts have a genetic organization different from that of the plant cells of which they are a part. All plant cells are eukaryotic and have their DNA in a membrane-bound nucleus, organized in complex chromosomes closely associated with histone proteins. Chloroplasts have a genetic organization similar to the simpler prokaryotes. Their DNA is a single circular molecule, with no nuclear membrane or histone proteins. Other similarities to prokaryotes include sequence homologies with E. coli DNA and prokaryote-type promoter regions (14). Chloroplasts do have some eukaryotic features as well, most notably the intervening sequences of DNA within some genes. Because chloroplast DNA and nuclear DNA have different organization, their regulation may differ (15).

MATERIALS AND METHODS

Plant growth

Pea (*Pisum sativum*) seeds were sterilized for ten minutes in a solution of 2.1% sodium hypochlorite and 0.25% Tween 20. They were then rinsed 5-10 times and soaked in distilled water for eight hours. The intact seeds were then placed in tubs containing moist vermiculite and covered with a thin layer of moist vermiculite. These tubs were then placed in light-sealed boxes and the germinating peas were watered as needed. After seven days of germination in the dark, the seedlings were exposed to light for 0, 4, or 24 hours (T_0, T_4, T_{24}).

Isolation and purification of RNA

Two grams of leaf tissue were collected from each set of seedlings. The tissue was frozen with liquid nitrogen and ground in a mortar. Kirby's solution was then added (3ml/2g leaf tissue), and the homogenate was transferred to a 30 ml Corex tube. This was followed by three phenol/chloroform extractions (5 ml phenol/chloroform added, mixed well, centrifuged (9800gx15min @ 4°C) and the top aqueous layer carefully removed). The volume of the final extract was recorded and the nucleic acids were precipitated by the addition of 1/20 volume 3M sodium acetate and 2.5 volumes absolute ethanol at -20°C.

The ethanol-precipitated nucleic acids were then centrifuged

(9800gx15min. @4°C). The pellet was then partially dried *in vacuo* . Two salt fractionations were performed to remove DNA by resuspending the pellets in 2 ml water and adding 3 volumes of 4M sodium acetate. This mixture was placed on ice for 2 hours. The samples were then centrifuged (17400gx15min @4°C) and the pellets were resuspended in 2 ml water. After the second salt fractionation, the RNA was percipitated in ethanol/sodium acetate as before.

The RNA samples were then DNased with *Stratagene* RNase free DNase according to the directions of the manufacturer. The samples were then re-extracted with phenol/chloroform as before to remove the enzyme. Aliquots of the samples were removed for analysis and the remainder was percipitated in ethanol/sodium acetate as before for storage. Spectrophotometric analysis of the samples using the absorbance at 260nm and 230 nm gave yeilds of 4.22 mg at T_0, 6.64 mg at T_4, and 5.53 mg at T_{24}.

Analysis of RNA

Denaturing formaldehyde (1% agarose/6.6% formaldehyde) gels were prepared according to Maniatis *et al* (16) with the RNA samples run at 20 and 40 micrograms per well. The RNA was blotted onto nitrocellulose according to standard procedures for a Northern transfer (16). Blots were dried and baked *in vacuo* for 2.5 hours prior to hybridization. A fragment

from the atpF gene (1.05 kb)(Figure 1) was obtained from Dr. Tsutsui and labeled with ^{32}P by the random prime method, using a Boehringer Mannheim kit and the instructions from the supplier. A labeled probe of the gene for the large subunit of ribulose bisphosphate carboxylase/oxygenase was obtained from Dr. John Mullet's laboratory (barley rbcL fragment, 1.5 kb). The membrane was then prehybridized for six hours at 42°C in 10 ml of prehybridization solution containing 50% formamide, 8x SSC, 5x Denhardt's, .05M sodium phosphate pH 6.4, and 1 mg denatured calf thymus DNA. The prehybridization solution was removed and the membrane was the hybridized at 42°C for 20 hours in 5 ml of the same solution with the radioactive probe added. The membrane was given 4 washes of 5 minutes each in 100 ml of 2x SSC with 1% SDS at room temperature. It was then washed twice for 15 minutes per wash in 100 ml of 0.1x SSC with 10% SDS at 50°C. The membrane was then exposed to x-ray film with an intensifying screen for three hours at -80°C for the rbcL gene and for three days for the atpF gene.

RESULTS

Results for the rbcL gene.

The photograph of the RNA hybridized to the rbcL gene probe (Figure 2) shows similiar levels of transcript in the T_0 and T_4 samples with an

increase in the amount of transcript in the T_{24} sample. This is visualized by the increased darkness of the bands of in the T_{24} samples. These results indicate that the 0 time duplicates at the lower level of RNA, (Panel A), show a single band of intensity higher than the 4 hour samples; this may have been caused by the inadvertent loading of both 0 time aliquots into the same well. The relative abundance of transcripts of this gene is photoregulated.

Results for the atpF gene

The photograph of the RNA hybridized to the atpF gene probe had no visible bands (data not shown). No results were obtained from this experiment.

DISCUSSION

The results for the rbcL gene were as expected. Light effects have been documented for the rbcL gene (2), so our results were not surprising. We were using this probe as a test to ensure that our RNA samples were adequately prepared and that our light conditions were satifactory, and to observe the kinetics of the light response of the rbcL gene. This chloroplast gene coded for the large subunit of ribulose bisphosphate carboxylase/oxygenase, the key enzyme of the Calvin cycle and a vital component of photosynthesis that is in high demand in photosynthetically

active plants. There were similiar levels of the transcripts at T_0 and T_4 with a dramatic increase in the levels in the T_{24} sample. This demonstrates the significant effect of light on messenger RNA accumulation in this problem. Kinetics studies have shown that hybridizable RNA transcripts for photoregulated genes increase in one of three ways; a sudden increase to a plateau, linearly, or linearly after a lag time (17). It seems that rbcL transcripts increase linearly after a lag time.

The results for the atpF probe were disappointing. We expect that the gene may be photoregulated because expression of several of the other subunits of the $cFoF_1$ ATP synthase have been shown to be controlled by light (3). Also, our experiment with the rbcL probe demonstrated photoregulation in a system known to show light effects, indicating that our experimental procedures were sound. The probe used for the Northern blot analysis did not incorporate as much radioactivity as we had expected, indicating that there might be less DNA in our original sample than we had anticipated or that the random prime labeling procedure for some unknown reason did not work with our 1.05 kb fragment. Photoregulation of the expression of the gene product, the F_0I subunit of the ATP synthase, was not demonstrated by our experiments; we intend to attempt the hybridization again with another DNA sample, however, to

determine if there is a light regulatory effect on the accumulation of this chloroplast mRNA.

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Figure I. Origin of pea chloroplast atpF fragment used as a hybridization probe in Northern blot analysis. The H 2.6 fragment of pea chloroplast DNA cloned into pUC 8 was restricted with DdeI/PvuII and the 1054 bp fragment was purified by electrophoresis on a 6% polyacrylamide gel.

Pea chloroplast DNA

H - HindIII P - PstI K - KpnI
E - EcoRI B - BamHI

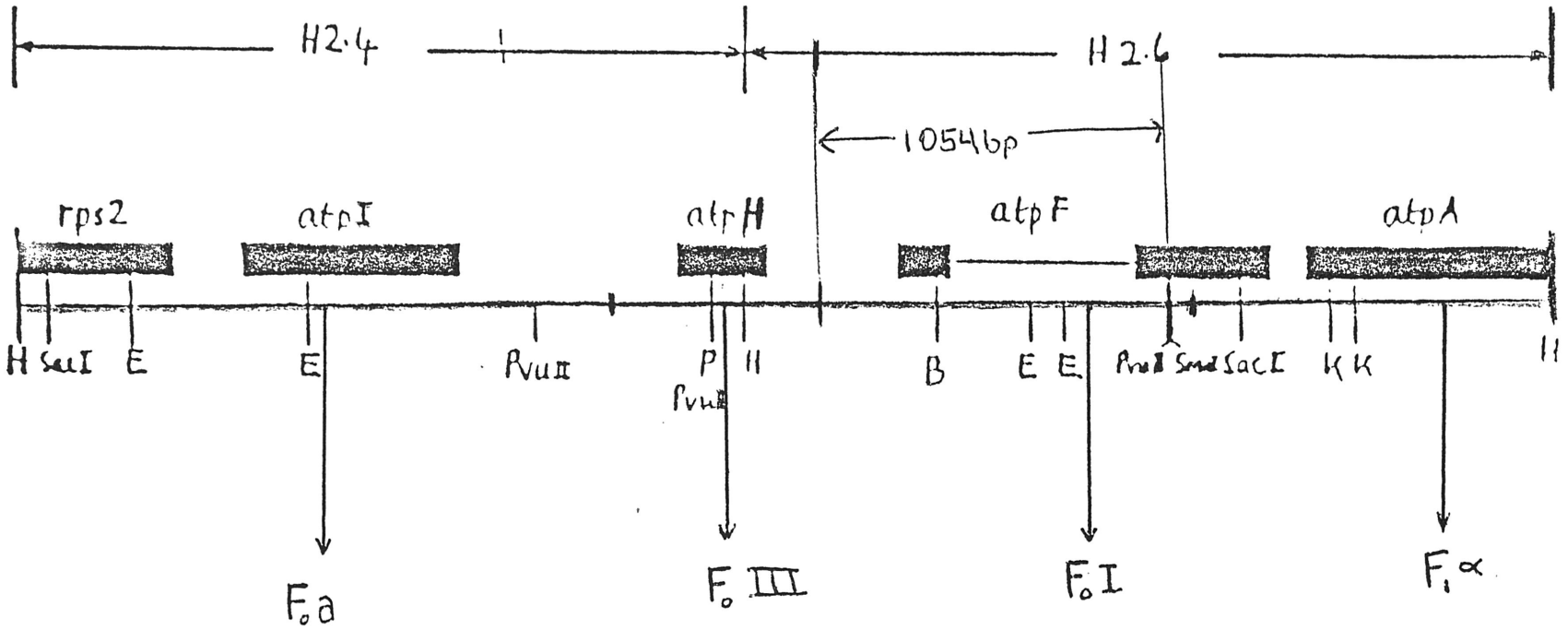
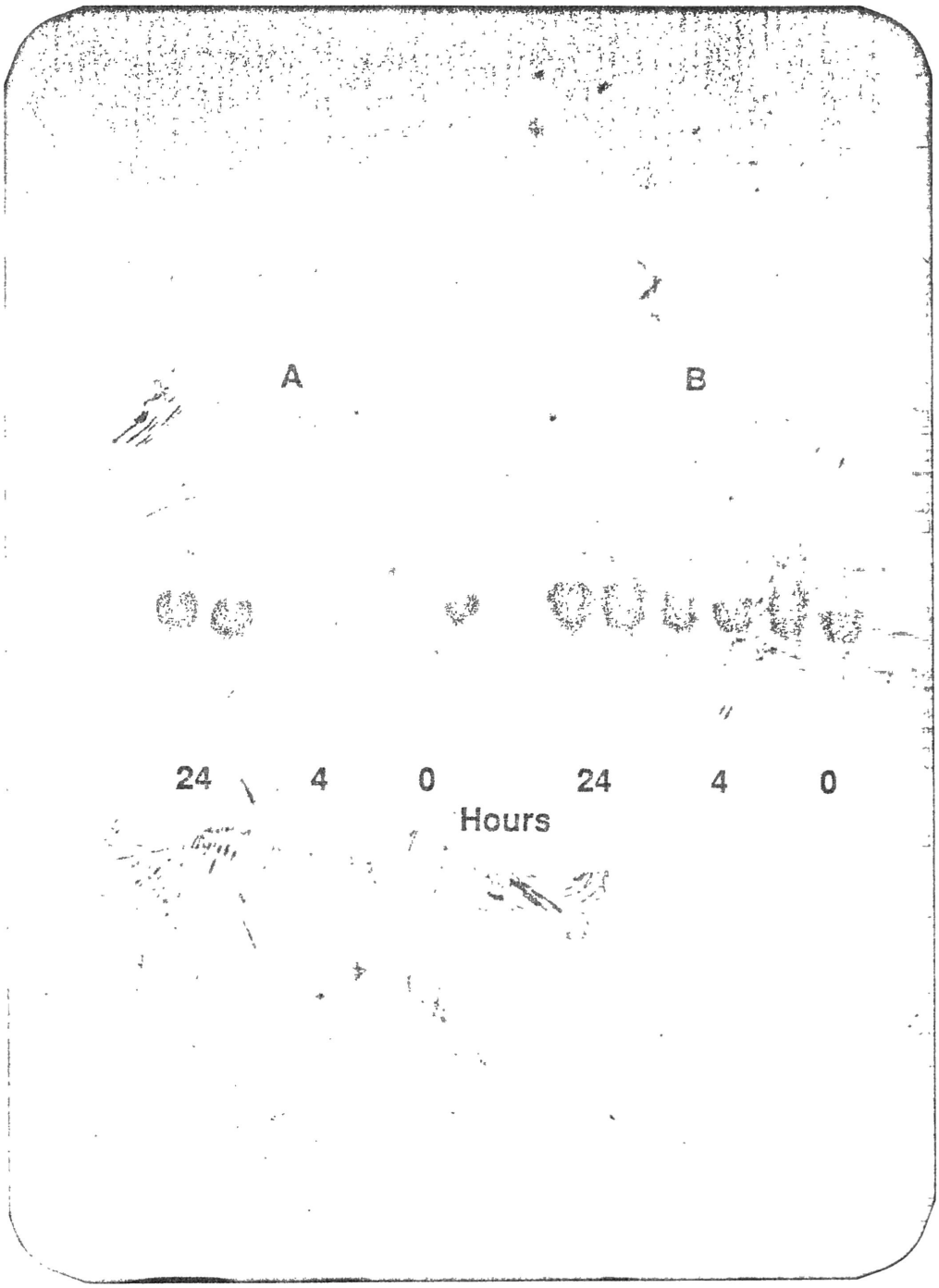


Figure 2. Autoradiographic image of a radiolabeled DNA probe for rbcL hybridized to a Northern blot of a 1% agarose/6% formaldehyde denaturing gel of total RNA extracts of pea leaf tissue. Panel A shows duplicate aliquots containing 20 μg of RNA per lane at light exposures of 0, 4, and 24 hours. Panel B shows duplicate aliquots containing 40 μg of RNA per lane at light exposures of 0, 4, and 24 hours.


A
B
24
4
0
24
4
0
Hours

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