THE EFFECT OF LIGHT ON EXPRESSION OF PEA CHLOROPLAST GENES

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ABSTRACT

The purpose of this research project was to determine whether light affects on the expression of certain chloroplast-encoded proteins of the pea ATP synthase complex. The proteins studied were the alpha and beta subunits of the CF_1 portion of the ATP synthase.

Dark-germinated pea seedlings were exposed to varying periods of light from 0 to 48 hours in length. Following light exposure, chlorophyll and total protein assays were run on harvested leaf tissue. The proteins in extracts of leaf tissue were separated by SDS polyacrylamide gel electrophoresis and then subjected to Western blotting. The synthase subunits were detected on the Western blot by reaction with specific rabbit antibodies; the antigen-antibody complex was visualized by a color reaction utilizing a second antibody enzyme conjugate.

Both the concentration of chlorophyll and of total protein in the leaf tissue rose with longer periods of light exposure. The alpha and beta subunits were both detected in the etiolated leaves of the seedlings which did not receive any light. The level of the beta subunit remained about the same until the seedlings had received 24 hours of light at which point the level showed a marked increase. At 48 hours of light, the beta protein level declined. However, the amount of the alpha subunit did not change noticeably with increasing light exposure.

INTRODUCTION

All organisms require some method of producing energy. In plants this method is photosynthesis. Photosynthesis traps light energy from the sun and produces carbohydrates from water and carbon dioxide. The light is trapped by a magnesium porphyrin molecule known as chlorophyll. Photosynthesis occurs in an organelle known as the chloroplast. The chlorophyll and the photosynthetic machinery is found in an internal membrane stacked upon itself known as the thylakoid membrane (Stryer, 1988). These thylakoid membranes account for as much as 90% of the cell membrane of plants (Gray, 1986).

Chloroplasts contain their own complement of genetic material or DNA. However, chloroplasts are not genetically independent. Only some of the subunits of the chloroplast polypeptide complexes are encoded in the chloroplast DNA, while the rest are coded for in the nucleus (Ohyama, 1988).

Chloroplast DNA (cpDNA) is not present in the same form as nuclear DNA. Instead cpDNA is found as circularized molecules which range in size from 85 kbp to more than 190 kbp. (Whitfield, 1983). Chloroplast DNA contains a set of large inverted repeats separated by a large single copy gene and a small single copy gene referred to as LSC and SSC respectively (Ohyama, 1988). This particular arrangement within the chloroplast genome is seen for almost all types of higher plants, with a few exceptions. The pea is one of the classes of higher plants which differ from the norm by not possessing an

inverted repeat portion (Kolonder, 1979).

The chloroplast DNA has been shown to contain all the chloroplast tRNA and rRNA genes, and it is expected that it contains all the genes for all the proteins synthesized in the chloroplast (Shinozaki, et.al. 1986).

Whether the genes of the plant are chloroplast or nuclear encoded, they are all subjected to environmental signals which may affect their expression. These environmental factors play a vital role in the development of a plant.

As a plant develops, chloroplasts are formed from small, immature organelles known as proplastids. These proplastids contain low amounts of plastid DNA, RNA, ribosomes, and soluble proteins. Proplastids develop into chloroplasts during maturation of the plant (Mullet, 1988).

In order for proplastids to fully develop, environmental factors must turn on the transcriptional and transnational machinery of the plant so that both chloroplast and nuclear genes may be expressed. One of these environmental factors is light. Light acts as a modulator of many of the developmental and regulatory mechanisms in higher plants (Tobin, 1985).

In order for light to induce developmental changes, the light itself must be trapped by photoreceptors found within the chloroplast. Higher plants have at least three photoreceptors which induce an expressional response to light. These photoreceptors include protochlorophyllide, phytochrome, and the blue light receptors (Tobin, 1985 and Anderson, 1987).

The blue light receptors are important in the regulation of mostly the lower plants and algae (Anderson, 1986).

Protochlorophyllide is eventually converted into chlorophyllide which is in turn is processed into chlorophyll. Not all the steps in this mechanism necessarily require light. Depending on the plant, the end product of chlorophyll can be synthesized in the dark, but not until critical primary reactions have taken place which require light (Anderson, 1986).

Before the protochlorophyllide is converted into chlorophyll, any leaf tissue that develops is immature and exhibits a pale yellow color. This color is due to a lack of mature chlorophyll in the plant tissue. Leaves in this state are said to be etiolated. Plastids of dark grown plants do not have mature chlorophyll and therefore their leaves are etiolated. The plastids of these dark grown plants are referred to as etioplasts (Mullet, 1988).

Phytochrome is known to participate in chloroplast development. A number of studies have shown that the expression of particular genes in higher plants are light regulated. The expression of both the nuclear and chloroplast encoded subunits of ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO), which catalyzes the fixation of carbon dioxide in plants, has been shown to be light dependant (Brioglie, 1984).

The study presented here looks at particular subunits of the ATP synthase complex in pea and their possible photoregulation.

During photosynthesis a proton concentration gradient is formed across the thylakoid membrane. Protons passing back through the membrane move through a proton channel in the CF_0F_1 ATP synthase and energize the formation of ATP in a process termed photophosphorylation (Stryer, 1988).

The ATP synthase is composed of two different domains. The CF_1 portion, an extrinsic complex, is the actual reaction center for photophosphorylation. It is composed of five protein chains known as alpha, beta, gamma, delta, and epsilon subunits. These are present in a stoichiometric ratio of 3:3:1:1:1 respectively. Figure 1 shows the structure of the CF_1 complex of ATP synthase.

The second portion, an intrinsic complex known as CF_0, provides the channel through the thylakoid membrane for the protons to pass through. The subunits of this complex are termed I,II,III, and IV. The appropriate stoichiometry of these CF_0 has not yet been worked out (Gray, 1988).

Of the nine subunits of the ATP synthase, six are plastid encoded. The plastid genes are: atpA (subunit alpha), atpB (subunit beta), atpE (subunit epsilon), atpF (subunit I), atpH (subunit III), and atpI (subunit IV) (Mullet, 1988). Of these six, atpF has been shown to contain a large intron which much be removed during processing (Bird, et.al. 1985).

Some of the ATPase plastid genes are contranscribed in a group. In higher plants, two main cotranscriptional clusters are found. One group contains the atpB and atpE gene. The other cluster contranscribes the atpA, atpF, and atpH genes

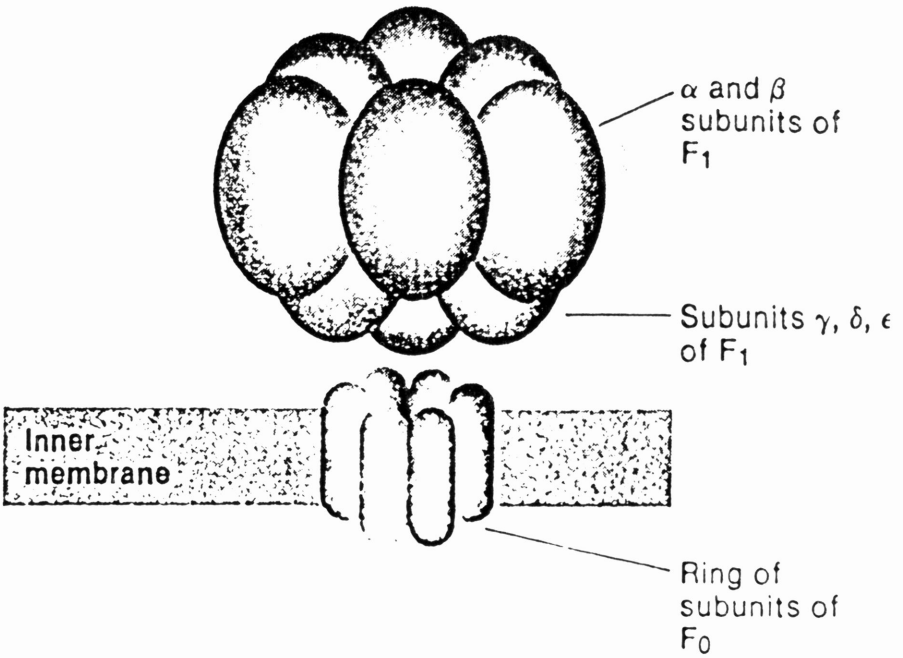

FIGURE 1: SUBUNIT STRUCTURE OF THE CF_0F_1 ATP SYNTHASE COMPLEX (Zubay, G., "Biochemistry", 2nd Edition, c1988, Macmillan Publishing Company, New York, p.553)

(Cozens, 1986).

The CF_1 alpha and beta subunits of the ATP synthase are the two proteins examined in this study. Dark-grown seedlings were exposed to different amounts of light and the proteins in each sample were analyzed using Western blotting procedures and immunological reactions. Rabbit antisera raised against wheat alpha and beta subunits was used in the immunological analysis. Antibodies against wheat could be used for the peas due to the high degree of homology between the subunits in higher plants.

A comparison of nucleotide sequences of the ATP synthase genes of different plants demonstrates a high degree of homology. in the case of the beta subunit a 95% homology is observed and a 90% homology is found in the alpha subunits across a wide spectrum of higher plants (Gray, 1988).

The alpha and beta subunits have been studied together because of their closeness in size. Since the two subunits differ in molecular weight by only 1 kDa they are difficult to separate and so the antiserum used contains a mixture of antibodies specific to each of the subunits. The size of the beta subunit is 57 kDa and that of the alpha subunit is 58 kDa (Gray, 1988).

MATERIALS AND METHODS

PEA GROWTH AND LIGHT EXPOSURE

Peas (Pisum sativum, Burpee variety: Progress no. 9) were first sterilized for five minutes in 100 ml of an aqueous solution containing 20ml of Chlorox bleach and one drop of Derma-Scrub liquid soap.

The peas were then planted in plastic cups, washed in Lysol, that contained 350 ml of moist vermiculite and placed in complete darkness for two weeks. Periodically the cups were removed in the presence of green light and watered to keep the vermiculite moist.

After fourteen days, the pea seedlings were moved into a growth chamber programmed for continual light. The day temperature in the chamber was $18^{0}C$, the night temperature was $20^{0}C$, and the light intensity was measured to be 175 microeinsteins M^{-2} sec^{-1}.

The seedlings were subjected to five different periods of light exposure: 0, 4, 8, 24, and 48hrs. The seedlings were moved into the growth chamber in timed intervals so that although their periods of light exposure were different, the actual growing time was the same overall. For example the 48 hr exposure was moved into the chamber first, followed a day later by the 24 hr exposure, etc.

After the appropriate period of light exposure, the leaf tissue from the seedlings was harvested, frozen in liquid nitrogen, weighted, and stored at $-80^{0}C$.

PROTEIN EXTRACTION

An extraction buffer of the following composition was prepared: 1mM EDTA (pH 8.0), 50 mM TRIS base (pH 7.5), 5mM dithiothreitol, 0.1M NaCl, and 0.5mM phenylmethylsulfonyl-fluoride. For each sample of leaves, 0.3g of tissue was ground in 0.6ml of extraction buffer in a mortar for five minutes at room temperature. The solution was transferred to Eppendorf tubes and centrifuged in a microfuge. The resulting supernatants were stored at -20^{o}C.

CHLOROPHYLL ASSAY (Arron, 1949)

For each sample of leaves, 0.1g of tissue was ground in a mortar with 1ml of 80% acetone for five minutes at room temperature. The solution was centrifuged for three minutes in a microfuge and the optical density of the supernatant was read at 663nm. The chlorophyll concentration of the leaf tissue was calculated using the extinction coefficient of chlorophyll at 663nm.

BRADFORD DYE-BINDING PROTEIN ASSAY

A protein standard curve was constructed using 0.1mg/ml bovine serum albumin in 0.1M NaOH as described by Marion Bradford (Bradford, 1976). For the sample extracts, a 10ul aliquot was taken and diluted to the appropriate volume with deionized distilled water for the micro assay procedure.

ELECTROPHORESIS AND BLOTTING

SDS polyacrylamide gel electrophoresis (SDS PAGE) was

performed as described by Sambrook et.al. (Sambrook, et.al. 1989). A Protean II mini-gel apparatus from Bio-Rad was used. Five microliters of each protein extract was loaded on a 12% gel. A mixture of protein size markers was also run. Following electrophoresis at 100V for an hour, protein bands on the gel were either stained with Coomassie Blue (Sambrook et.al., 1989) or blotted onto nitrocellulose (Western blot) as described by Sambrook et.al (Sambrook, et.al. 1989). The gel was stained with Coomassie Blue after blotting to ensure that proper protein transfer to the nitrocellulose membrane had occurred.

IMMUNOBLOT STAINING

The membrane was probed with rabbit antibodies raised to the alpha and beta subunits of CF_1 according to the directions of Sambrook et.al. (Sambrook, et.al. 1989). The antiserum was kindly provided by Dr. John C. Gray of Cambridge University, England. The second antibody was goat antirabbit IgG conjugated to horseradish peroxidase from Bio-Rad Laboratories. The antigenic alpha and beta subunits were visualized on the filter as bluish-purple bands which arose from the reaction of 4-chloro-1-naphthol and hydrogen peroxide catalyzed by the peroxidase conjugated to the second antibody.

RESULTS

The results of the chlorophyll and Bradford protein assays are summarized in Table 1.

For the 8 hour exposure, the leaves were starting to open up, and traces of green could be found. The 24 and 48 hour light exposures, however, had fully open and green leaves.

The length of light exposure was not a factor in the amount of tissue harvested. This amount was determined simply by the number of peas in each cup that germinated and grew sufficiently so that leaves could be cut from the stems. In most of the samples, well over 50% of the peas germinated and some tissue could be removed.

Using the Beer-Lambert law and the extinction coefficient for chlorophyll at 663nm, the concentration of chlorophyll was determined. The values are presented in grams-chlorophyll/gram of leaf tissue. As seen in Table 1, the concentration of the chlorophyll increased as the length of light exposure increased. There was a ten-fold increase in this concentration in the first eight hours of light exposure and a further four-fold increase between 24 and 48 hours. Increasing amounts of chlorophyll correlate with the development of chloroplasts by the light-exposed seedlings. Chlorophyll synthesis requires light. As chlorophyll increases in the leaves, etioplasts are developing into fully functional chloroplasts capable of photosynthesis.

Total protein concentrations in the leaf tissue were calculated by comparison with the protein standard curve. As

LIGHT EXPOSURE (hrs)	CHLOROPHYLL (g/g leaf tissue)	TOTAL PROTEIN (g/g leaf tissue)
0	1.01 x 10-4	0.021
4	3.57 x 10-4	0.031
8	1.09 x 10-3	0.038
24	1.25 x 10-3	0.048
48	4.51 x 10-3	0.056

TABLE 1: CHLOROPHYLL AND TOTAL PROTEIN CONTENT OF PEA LEAF TISSUE EXPOSED TO LIGHT FOR VARYING PERIODS OF TIME

with the chlorophyll assay, the concentrations are expressed in grams total protein/gram of leaf tissue. As expected, the total amount of protein in the developing leaves steadily increased as the length of light exposure increased.

A photocopy (xerox) of the SDS PAGE gel of the leaf protein extracts is shown in Figure 2. The lanes are marked according to the sample they held, and the sizes of the protein markers are indicated. By studying individual bands, two very important trends can be seen. The first is that the intensity of certain specific bands seems to increase as as the length of light exposure increases. A darker band across a sample indicates a larger amount of protein. This is seen prominently in a band located at approximately 15 kD. This trend is also seen at roughly 13kD where a band was not even detectable on the gel until the seedlings had been exposed to twenty four hours of light. There is a thick band that follows this trend too at approximately 55kD. This thick band could be the large subunit of RUBISCO since it is the most prominent protein quantitatively speaking in plants. The actual identity of this band was not determined however.

Another trend can be observed. Some bands reach their greatest intensity before 48 hours of light exposure, and then decrease in intensity with further illumination. Such a trend can be seen with bands at approximately 16kD and at 20kD; these bands were much easier to see in the original gel. This may indicate that the rate of synthesis of the protein has

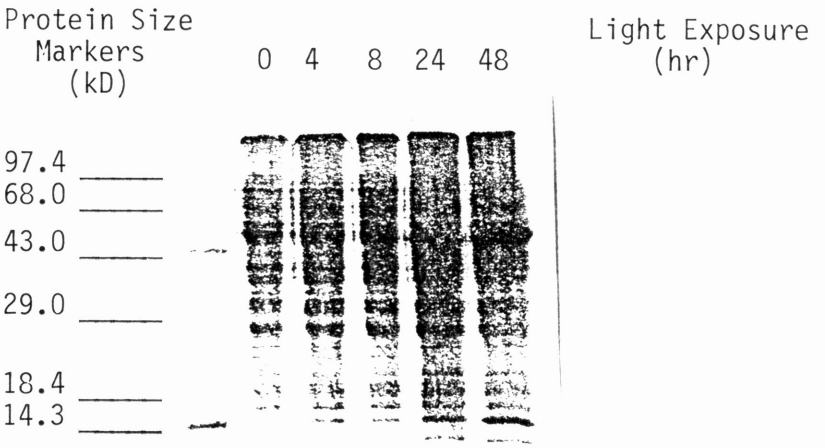

FIGURE 2: SDS POLYACRYLAMIDE GEL OF TOTAL LEAF PROTEIN STAINED WITH COOMASSIE BLUE R-250

decreased, or that the protein is being degraded with time.

The concentration of the alpha and beta subunits in the leaf extracts were too small to be detectable by Coomassie Blue staining (sensitivity in the microgram range), so a much more sensitive and specific antigen-antibody reaction (sensitivity: 250-500 picograms) was used to detect the subunits after Western blotting of the proteins onto a nitrocellulose membrane. A photocopy (xerox) of the immunoblot is seen in Figure 3. The length of light exposure is indicated for each of the sample lanes. (The extraneous markings in the figure are the results of unavoidable wrinkles in the saran wrap in which the blot was wrapped for xeroxing). The result of the actual immunoblot and color reaction was a sheet of pure white nitrocellulose with two distinct rows of bluish/purple bands. Since the beta subunit is the smaller of the two (see introduction), it is the more prominent band farther down on the membrane. A thin band, which is apparent just above the beta subunit band corresponds to the alpha subunit.

On the actual blot, the intensity of the bands was much more visible than on the xerox copy. It is difficult to discern any differences in the intensity of this alpha band with only the naked eye. At 24 hours, however the beta subunit band is much more intense than the relatively constant bands at 0,4, and 8 hours, while at 48 hours, the intensity has decreased noticeably. By 24 hours of exposure to light, the concentration of the beta subunit has noticeably increased, but the decreased intensity at

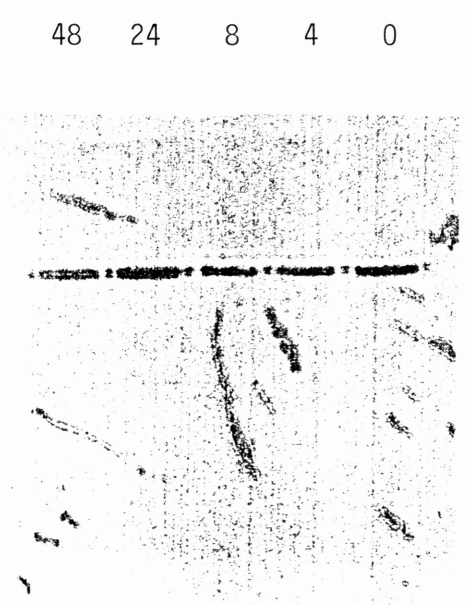

FIGURE 3: IMMUNOBLOT OF TOTAL PEA LEAF PROTEIN REACTED WITH ANTISERUM TO WHEAT CF_1 ALPHA AND BETA SUBUNITS

48 may be due to either decreased synthesis or increased breakdown of the protein.

DISCUSSION

From the data obtained, the expression of at least the beta subunit of the dicot pea ATP synthase does seem to be light regulated to some extent. The results indicate that light is not needed for the preliminary expression of these subunits, but it is needed to stimulate increased production as the chloroplast develops and becomes fully functional. However, after a certain point, probably the complete maturation of chloroplasts, the rate of increasing synthesis drops off and/or the proteins are degraded.

These results are comparable with other studies. ATP synthase has been found along with other chloroplast proteins in higher plants grown in the dark (Gray 1986). Therefore light is not a primary requirement for the initial synthesis of these chloroplast proteins. However, it has been shown that during plastid development (i.e. the conversion of etioplasts into fully functional chloroplasts) in spinach, the transcription activity of the mRNA for the plastid genome including subunits of ATPase increases and then decreases as maturation is reached (Deng, 1987). If the mRNA levels rise and then fall off, it would not be surprising to see the protein concentrations do the same.

For further studies, the amounts of the alpha and beta subunits should be studied at light exposures that concentrate on

the times between eight and forty- eight hours. This would help in identifying specifically the time when the concentration begins to increase and when this concentration reaches its peak. It would also be interesting to determine if the increase in the concentration of subunits shows a gradual rise over the time between eight and twenty-four hours or if there was a sudden, large change within one period of time.

In a longer and more detailed study, the mRNA for these subunits should be isolated and studied. Such a study would then involve a comparison between the rate of increase in transcription versus translation for these subunits.

Finally, for any further investigation, alkaline phosphatase should be used in the immunological color reaction instead of horseradish peroxidase. Horseradish peroxidase color is very unstable in light. The color observed on the nitrocellulose membrane fades dramatically if exposed to only a few minutes of light. After making several xerox copies to provide a permanent record, the bands showed a drastic decrease in intensity.

This instability made density measurements of the bands on a computing densitometer impossible. Density measurements of the bands would have been extremely helpful for the 0,4, and 8 hour samples, since the protein concentration may actually be increasing but not to the extent that can be noticed simply by the naked eye. Color reactions using alkaline phosphatase would provide a more stable product for quantitative analysis.

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REFERENCES

Anderson, Jan (1986). Photoregulation of the Components, Function and Structure of Thylakoid Membranes. Annual Review of Plant Physiology 37: 93-136.

Arron, D. (1949). Copper enzymes in isolated chloroplasts. Plant Physiology 24: 1-15.

Bird, C.R.; Koller, B.; Auffret, A.; Huttly, A.; Howe, C.; Dyer, T.; and Gray, J.C. (1985). The wheat chloroplast gene for CF_0 subunit I of ATP synthase contains a large intron. The EMBO Journal 4, #6: 1381-1388.

Bradford, Marion (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Broglie, Richard, Coruzzi, G., Fraley, R., Rogers, S., Horsch, R., Niedermeyer, J., Fink, C., Flick, J., and Chua, Nam-Hai. (1984) Light-regulated expression of a pea ribulose-1,5-bisphosphate carboxylase small subunit gene in transformed plant cells. Science 224: 838-843.

Cozens, Alison; Walker, John: Phillips, Andrew; Huttly, Alison; and Gray, John C. (1986). A sixth subunit of ATP synthase, an F_0 component, is encoded in the pea chloroplast genome. The EMBO Journal 5, #2: 217-222.

Deng Xi, and Grusisan A. (1987). Control of plastid gene development: transcripts of atp BE operon increases and then decays. Cell 49: 379-395.

Gray, John; Bird, C.; Courtice, G.; Hird, S.; Howe, C.; Huttly, A.; Phillips, A.; Smith, A.; Willey, D.; Bowman, C.; and Dyer, T. (1986). Chloroplast genes for photosynthetic membrane components of higher plants. Biochemical Society Transactions 14: 25-27.

Gray, John. (1987) Genetics and synthesis of chloroplast membrane proteins. Photosynthesis, J Amesz ed., Elsevier Science Publishers:319-342.

Hughes, J.E. and Link, G. (1988) Photoregulation of psb A transcript levels in mustard cotyledons. Photosynthesis Research 17: 57-73.

Kolonder, Richard (1979) Inverted repeats in chloroplast DNA from higher plants. Proceedings of the National Academy of Sciences U.S.A. 76 #1: 41-45.

Kuhlemire, Chris. (1987) Regulation of gene expression in higher plants. Annual Review of Plant Physiology 37: 93-136.

Mullet, John (1988) Chloroplast development and gene expression. Annual Review of Plant Physiology and Plant Molecular Biology 39: 475-502

Ohyama, Kanji, Kohchi,T., Sano, T., and Yamada, Y. (1988) Newly identified groups of genes in chloroplasts. TIBS 13: 19-22.

Sambrook, J.; Fritsch, E.; and Maniatis, T. (1989) Molecular Cloning: A Lab Manual 2nd ed, Cold Spring Harbor Publishing.

Shinozaki, K.; Ohme, M.; Tanaka, M.; Wakasuge, T.; Hayashida, N.; Matsubayashi, T.; Zaita, N.; Chunwongse, J.; Obokata, J.; Yamaguchi-Shinozaki, K.; Ohto, C.; Torazaua, K.; Meng, B.; Sugita, M.; Deno, H.; Kamogashira, T.; Yamada, K.; Kusada, J.; Takaiwa, F.; Kato, A.; Tohdoh, N. Shimada, H.; and Sugiura, M,; (1986) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. The EMBO Journal 5, #9: 2043-2049.

Stryer, Lubert. (1988). Biochemistry. W.H. Freeman and Co, NY.

Tobin, Elaine and Silverthorne, Jane (1985) Light regulation of gene expression in higher plants. Annual Review of Plant Physiology 36: 569-582.

Whitfield, A. (1983). Organization and structure of chloroplast genes. Annual Review of Plant Physiology 34: 279-310.