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Purification, characterization and light regulation of pea chloroplast protein synthesis elongation factor G: Identification and partial sequencing of its gene

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PURIFICATION, CHARACTERIZATION AND LIGHT REGULATION OF PEA CHLOROPLAST PROTEIN SYNTHESIS ELONGATION FACTOR G; IDENTIFICATION AND PARTIAL SEQUENCING OF ITS GENE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

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PUBLICATIONS

Breitenberger, C. A.; Akkaya, M. S.; Duerr, B. K.; Welcsh, P. L. "Light Regulation of Pea Chloroplast Protein Synthesis Factor EF-G Activity." The FASEB Journal **4** (1990) A1844.

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FIELDS OF STUDY

Major Field: Biochemistry

Studies in Protein Purification and Characterization and Molecular Biology of Plants.

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AIU:	Arbitrary intensity unit
ASF:	Ammonium sulfate fractionate
ATP:	Adenosine triphosphate
BCA:	Bicinchoninic acid
bp:	Base pair
ß-ME:	Beta-mercaptoethanol
CTP:	Cytidine triphosphate
cDNA:	Complementary DNA
chl:	Chloroplast
chlEF-G:	Chloroplast Elongation Factor G
chlEF-Ts:	Chloroplast Elongation Factor Ts
chlEF-Tu:	Chloroplast Elongation Factor Tu
cpm:	Counts per minute
CTP:	Cytidine triphosphate
dATP:	Deoxyadenosine triphosphate
dCTP:	Deoxycytidine triphosphate
[^{14}C]-Phe:	[^{14}C]-phenylalanine
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
EF-G:	Elongation Factor G
EF-Tu:	Elongation Factor Tu
EF-Ts:	Elongation Factor Ts
FAP:	Fusidic acid affinity purified
EFs:	Elongation Factors
HPLC:	High performance liquid chromatography
hr(s):	Hour(s)
hν:	Light
hν↑:	Light-induced
hν↓:	Light to dark transferred
-hν:	Dark-grown
+hν:	Light-grown
IF:	Initiation factor
kbp:	Kilobase pair
kD:	Kilo Dalton
LSU:	Large subunit of Rubisco
M:	Molar
min:	Minute
mL:	Milliliter
mM:	Millimolar
μg:	Microgram
μm:	Micrometer
mRNA:	Messenger RNA
MOPS:	(3-[N-Morpholino]propane sulfonic acid)

mito:	Mitochondrial
ng:	Nanogram
PMSF:	Phenylmethyl sulfonyl fluoride
PNK:	Polynucleotide Kinase
PRS:	Postribosomal supernatant
PS I:	Photosystem I
PS II:	Photosystem II
RNA:	Ribonucleic Acid
rpm:	Revolution per minute
Rubisco:	Ribulose-1,5-bisphosphate Carboxylase/Oxygenase
sec:	Second
SDS:	Sodium Dodecylsulfate
SSU:	Small Subunit of Rubisco
tRNA:	Transfer RNA

CHAPTER I

INTRODUCTION

The protein synthesizing machinery of higher plant chloroplasts bears a high resemblance to prokaryotic protein synthesis systems. However, most of the components of this system are encoded by nuclear genes, synthesized in the cytoplasm with a specific N-terminal transit peptide and then transported into the chloroplast. It would be very difficult to rationalize these observations without an evolutionary perspective.

The similarity between chloroplasts and free-living blue-green algae was first noted by nineteenth century microbiologists (Altmann, R., 1890). However, the idea of organelles inside eukaryotic cells being symbionts of bacterial origin was received with considerable criticism. Only after 1964, when DNA was found in chloroplasts (Ris, et al., 1962) and mitochondria of plants and animals (Bell, et al., 1964; Luck, et al., 1964; Nass, et al., 1965), was this idea taken more seriously. Today, prochloroplast and promitochondrial organisms are thought to have cohabited the eukaryotic lineage leading to higher plants where they managed to survive endosymbiotically. Furthermore, as these symbiotic

relationships evolved, DNAs had been redistributed between the organelles and the nucleus of such organisms to the point that some DNA may had been transferred to nuclear DNA, or totally lost.

The chloroplast is the most obvious candidate for an organelle of symbiotic origin, and it is likely to have been acquired relatively recently compared to mitochondria. The chloroplasts of the green alga *Chlamydomonas reinhardii* code for several hundred proteins, in contrast to the mitochondria which have about one-tenth of that capacity (Margulis, 1981). But, apparently the chloroplasts have transferred some genetic information to the nuclear chromosomes of the host, because participation of both nuclear and chloroplast genes is required for biogenesis. For example, a chloroplast gene codes for the large subunit of the enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), but in many plants, the gene coding for small subunit is found in a nuclear chromosome. This kind of cooperation is indicative of an ancient and now obligate symbiotic relationship. For the most part then, the organelles became dependent upon the nuclear gene products for their own biogenesis. Among the gene products of chloroplasts that are nuclear encoded are subunits of Rubisco, PSI, PSII, ATP synthetase, cytochrome b/f complex, and some of the subunits of the replication, transcription, and translation apparatus required for chloroplast biogenesis. More than 90 % of the mitochondrial and less than 90 % of the

chloroplast proteins are encoded on nuclear DNA (Gray, 1989, Attardi et al., 1988, Sugiura, 1989, Taylor, 1989). Therefore chloroplast biogenesis and gene regulation must be at least partly under the control of nuclear events. This presumably requires a complex coordination mechanism among chloroplast, nuclear and cytoplasmic events, light may be playing a major role in coordination.

The higher plants have at least three major photoreceptors; phytochrome, protochlorophyllide and blue-light receptors. Phytochrome absorbs red and far-red light, and exist in two spectrophotometric forms; the red-absorbing, (P_r), inactive form and the far-red-absorbing, (P_{fr}), active form. These two forms are interconvertible by irradiation at red and far-red wavelengths through isomerization of the chromophore, tetrapyrrole biliverdin (Rudiger, 1987). Absorption of red light by P_r activates it by converting to P_{fr} and far-red light reverses this process. Therefore, red light irradiation followed by far-red light irradiation is usually used to determine if phytochrome is involved in light-regulated events. The two forms of phytochrome also absorb in the blue-light region of the spectrum, and to some extent, at other wavelengths too. Protochlorophyllide, which is the precursor of chlorophyll a/b pigments, is involved in photosynthetic apparatus and formation of chloroplasts. Conversion of protochlorophyllide into chlorophyllide requires a specific enzyme and light (Griffiths, 1978 and Thorne,

1971). Because the light absorbed by protochlorophyllide overlaps with the phytochrome light absorbing region, far-red reversibility of phytochrome is the criterion for differentiating the operations of these receptors from each other. The photoreceptors interact with each other, in some cases these interactions are required for maximum levels of gene expression, genes of large subunit of Rubisco (LSU) and chlorophyll a/b binding protein (CAB) (references cited in Thompson, 1991).

Photoregulation of chloroplast biogenesis:

Functional chloroplasts develop from proplastids, which lack pigmentation (chlorophylls) and internal membranes. As plants develop in the presence of light, chlorophylls are accumulated and thylakoid membranes develop fully. Light is probably the most important environmental stimulus for plants. In addition to its direct involvement in photosynthesis, light also has regulatory effects on the expression of certain genes and overall developmental processes of plants. Figure 1 shows the major differences between light-grown and dark-grown peas. Note the difference in stem elongation, leaf development, and color; dark-grown peas are pale yellow, light-grown peas are green.

The chloroplast and nuclear genomes are both involved in the chloroplasts biogenesis, although the chloroplast encodes its own rRNAs and a set of tRNAs (Shinozaki et al., 1986)

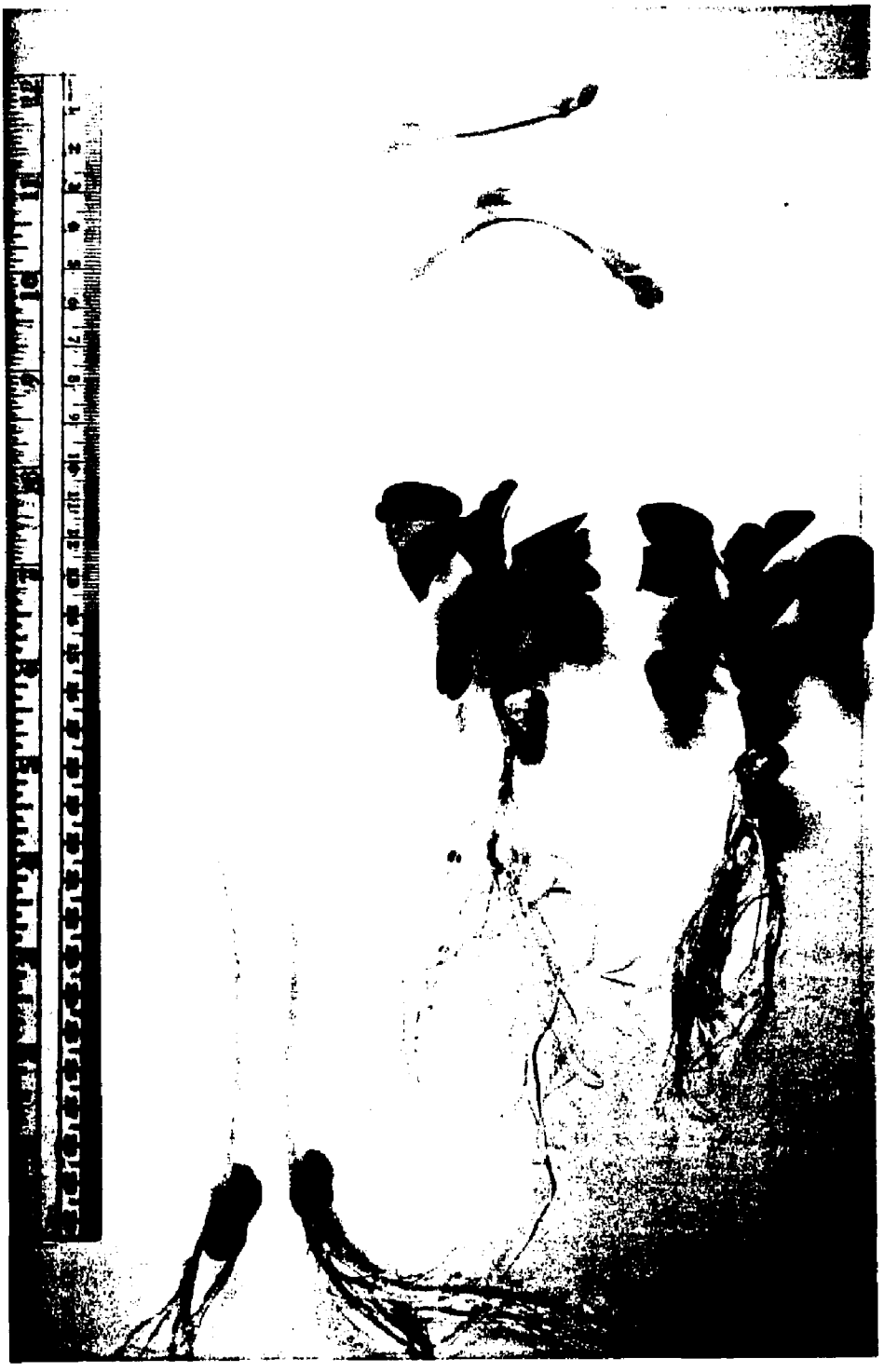


Figure 1: 13 days dark- and light-grown peas.

Chloroplast biogenesis is induced by light and this induction increases the overall protein synthesis capacity which is very low in the plastids (etioplasts) of dark-grown plants (Reger et al., 1972, Siddel et al. 1975). Many chloroplast genes seem to be transcribed constitutively in the dark and in the light. Many genes encoded by the chloroplast genome are regulated by light at the posttranscriptional level, *e.g.*, the large subunit of Rubisco (LSU). The increase of LSU mRNA levels in etiolated peas is only three-fold upon exposure to light, however LSU protein levels increase 50 times. The increase in mRNA can be accounted for by the three-fold increase in DNA copy number in the light (Inamine et al., 1985). In petunia too, the regulation of this gene is at the translational level (van Grinsven et al., 1986). However, the gene for Rubisco LSU in barley appears not to be regulated at the translational level (Mullet et al., 1985). In barley it was demonstrated that plastid protein synthesis is stimulated by red or white light, also far-red light partially reversed the red-light effect (Gamble et al., 1989). This suggests phytochrome is one photoreceptor involved in light regulation of protein synthesis of chloroplasts. The general transcription of plastids also increases after illumination, in some cases the increase in mRNA levels does not correlate with plastid DNA copy number. For example accumulation of the mRNA for 32 kd quinone-binding protein of photosystem II is stimulated to a great extent by light in

some plants (Grebanier et al., 1978). Nevertheless, it was shown that posttranscriptional regulation is a much more effective control than the transcriptional regulation in plastid gene expression (Deng et al, 1987). It seems that the increase in transcription of some light induced chloroplast genes is more likely a function of light induced development of chloroplasts than a direct light effect on those specific genes (Tobin et al., 1985).

Ellis (Ellis, 1977) in his proposal of the "cytoplasmic control principle" indicated that cytoplasmic gene products control transcription and translation of gene products in plastids since organellar gene expression was arrested when cytoplasmic protein synthesis was inhibited. Recently, it was shown that inhibition of chloroplast protein synthesis can cause an inhibition of nuclear gene production as well (Bonen et al., 1986 and Oelmuller et al., 1986), suggesting an even more complex coordination mechanism. Coordination of protein synthesis and assembly of chloroplast proteins requires co-activation of nuclear and chloroplast gene transcription and translation by environmental and cellular signals.

The light regulation of genes that are encoded by the nuclear genome and transported into the chloroplast is mostly at the level of transcription; *e.g.*, small subunit of Rubisco (Tobin et al., 1985), and chlorophyll a/b binding protein (Nagy et al., 1986). Rubisco is one of the best studied examples of light regulation of gene expression in higher

plants. This enzyme is present in chloroplasts, and is responsible for CO_2 fixation in photosynthesis. It is a multimeric protein with 8 large and 8 small subunits. The small subunit is encoded by nuclear DNA and synthesized on cytoplasmic ribosomes as a precursor, with a transit peptide on the N-terminus. The small subunits of Rubisco (SSU) are then imported into the chloroplast where they are assembled into functional Rubisco. The large subunits of Rubisco (LSU) are encoded by the chloroplast genome and translated by the chloroplast protein synthesizing system. The expression of SSU and LSU were found to be light regulated at the transcriptional and the translational levels, respectively, in pea (Sasaki et al., 1981; Thompson et al., 1983 and Inamine et al., 1985). The mRNA of SSU appears to be light-regulated by the phytochrome receptor at the level of transcription initiation in tobacco (Tobin et al., 1985). In pea, accumulation of SSU mRNA is not very rapid and there are two light receptors required for the maximum level its expression (Fluhr et al., 1986). They showed that red light treatment induces the SSU gene expression in dark-grown peas with little effect on dark-adapted peas, blue light however could induce the transcription level of this gene to a great extent in the dark-adapted peas. Red-light treatment following blue-light reduces the blue light effect, suggesting a co-operation of the blue light receptor and phytochrome. Therefore, red light responses of certain genes are different in the green plants

and etiolated plants. There is a strong phytochrome response of SSU and CAB genes in etiolated plants. However, in greening pea shoots, red to far-red ratio does not have an effect on the abundance of these gene transcripts (Jenkins et al., 1985).

In summary, photoregulation of plastid and nuclear genes (for plastid proteins) is not only interlinked with development, but also the level of regulation seems to vary for the same genes among the different plant species, even in different tissue and organs in one species (Taylor, 1989).

Protein synthesis is in general controlled in both prokaryotic and eukaryotic systems at the level of initiation. For example, in immature red blood cells the activity of IF-2 was reduced in a controlled way by phosphorylation of one of its three protein subunits, suggesting a control in part by some specific protein kinases (Ochoa et al., 1979). (Phosphorylation or dephosphorylation of EF-2s in some cells are discussed in the Chapter III, part 3.3). The control of chloroplast protein synthesis at the elongation step in translation by EF-G is proposed in Chapter V. Ribosomal protein S6 is phosphorylated in mammalian cells. The extent of phosphorylation, which is induced by hormones, cAMP, etc.; parallels the rate of growth (Traugh et al., 1986). In general then, proteins associated with mRNAs may specifically repress or enhance their translation by some allosteric or covalent modification mechanisms (Rosenthal, et al., 1980).

In bacteria, there is found to be a translational feedback mechanism. Excess amounts of ribosomal proteins inhibit their own synthesis by binding to specific sequences on the 5' end of the mRNA (Dean, et al., 1980).

Euglena gracilis (E. gracilis) is an unicellular photosynthetic eukaryotic organism. The chloroplast of this organism is different from those of higher plants (*E. gracilis* chloroplasts and higher plant chloroplasts probably evolved from distinct endosymbiotic events (Gray, 1989)). Unlike higher plants, the thylakoid membranes in *E. gracilis* are not stacked in grana, instead they form divisions. A third membrane is present in *E. gracilis* which may be extended from the endoplasmic reticulum (Lefort-Tran, 1981). In addition to the differences in structural features, *E. gracilis* chloroplasts are not essential for life. In the dark, cells can shift from phototrophic to heterotrophic growth, chloroplasts in this condition become vestigial proplastids. However, functional plastids are essential in higher plants.

Genetic information for some components of the chloroplast protein synthesizing system was obtained from a mutant *E. gracilis* strain which loses most of its chloroplast DNA upon UV-irradiation (Hecker et al., 1974). The gene for *E. gracilis* chloroplast elongation factor G was shown to be located in the nuclear genome (Breitenberger et al., 1979b). The evidence was obtained from studies with inhibitors specific for cytoplasmic and chloroplast protein synthesis

systems and with a mutant strain of *E. gracilis*, (W_3BUL, mentioned above). Cycloheximide, a specific inhibitor of cytoplasmic protein synthesis on cytoplasmic ribosomes, inhibited light induction of chlEF-G in the *E. gracilis*. Spectinomycin or streptomycin (inhibitors of chloroplast protein synthesis) did not have any effect on the induction. Also, chlEF-G was partially induced by light in the mutant strain.

In *Euglena gracilis*, chloroplast protein synthesizing capacity is stimulated 140 times by growth in the light. Chloroplast protein synthesis elongation factors have been shown to be light regulated; EF-Ts (Fox et al., 1980), EF-Tu (Sreedharan et al., 1985), IF-2 (Gold et al., 1985), IF-3 (Kraus et al., 1986) and EF-G (Breitenberger et al., 1980) much more than others. ChlEF-G is at least partly responsible for the induction of the protein synthesis capacity (Breitenberger et al., 1979b).

Thus, my project starts with the understanding of the stimulating effect of light on chloroplast protein synthesis. Because chlEF-G is the most strongly light regulated component in *E. gracilis* chloroplast protein synthesis, it is likely to be at least partly responsible for the induced capacity of chloroplast protein synthesis by light in higher plants. ChlEF-G is nuclear genome encoded, it may also play a very important role in coordination of chloroplast and nuclear events in a light-dependent manner.

By working with pea, we investigated the light regulation of chloroplast EF-G in higher plants. We were successful in purification of chlEF-G to homogeneity and in obtaining N-terminal amino acid sequence and part of the cDNA sequence.

CHAPTER II

PURIFICATION AND CHARACTERIZATION OF PEA ORGANELLAR EF-G'S

2.1 Introduction

As discussed in detail in the next chapter, we have found that chlEF-G activity is induced by light, and this regulation may be phytochrome-mediated. It was very difficult to determine the magnitude of this induction, since the extracts of dark grown samples resulted in no activity at some time points and very low residual EF-G activities in others. Therefore, I initiated an attempt to purify EF-G both from dark-grown and light-induced pea seedlings. The EF-G activity is rapidly light-induced upon transfer of 13 days dark-grown pea seedlings to light for 3 days, resulting in at least 2-fold activity increase. Therefore, chloroplast EF-G was isolated from pea seedlings which were 13 days dark-grown followed by 3 days light exposure. EF-G was partially purified from 13 day old dark-grown peas as well.

Chloroplast EF-G was originally purified from isolated chloroplasts of spinach (Tiboni et al., 1978). In order to bypass difficulties and variables associated with the large scale chloroplast isolations, we preferred to use total cell

extracts, in combination with an affinity binding procedure. This purification took advantage of the affinity of organellar EF-G for prokaryotic ribosomes. This method was previously used for chloroplast and mitochondrial EF-G purification from *E. gracilis* (Breitenberger et al. 1979a and Eberly et al., 1985) and was originally developed for purification of *E. coli* EF-G (Rohrbach et al. 1974). We have adapted the procedure for pea with very minor modifications. The procedure (Figure 2) employs fusidic acid, a steroidal antibiotic, which stabilizes a [translocase . GDP . ribosomal] complex. This stable complex can be isolated by high speed centrifugation and then dissociated by a high salt wash of the ribosomal complex. Cytoplasmic protein synthesis elongation factor 2, EF-2, does have an affinity for eukaryotic ribosomes, but not for prokaryotic ribosomes with which the ribosomal complex is formed in this purification method (for purification of *E. gracilis* EF-2, wheat germ ribosomes were used in Breitenberger et al., 1979a). Therefore, this technique readily eliminates a large body of postribosomal proteins as well as a cytoplasmic protein synthesis elongation factor, EF-2.

The activity assay only measures the activity of organellar elongation factors, and not cytoplasmic factors, since organellar EF-Gs are interchangeable with prokaryotic EF-Gs for protein synthesis on prokaryotic ribosomes (Grivell et al., 1972). Only organellar EF-Gs can act as functional

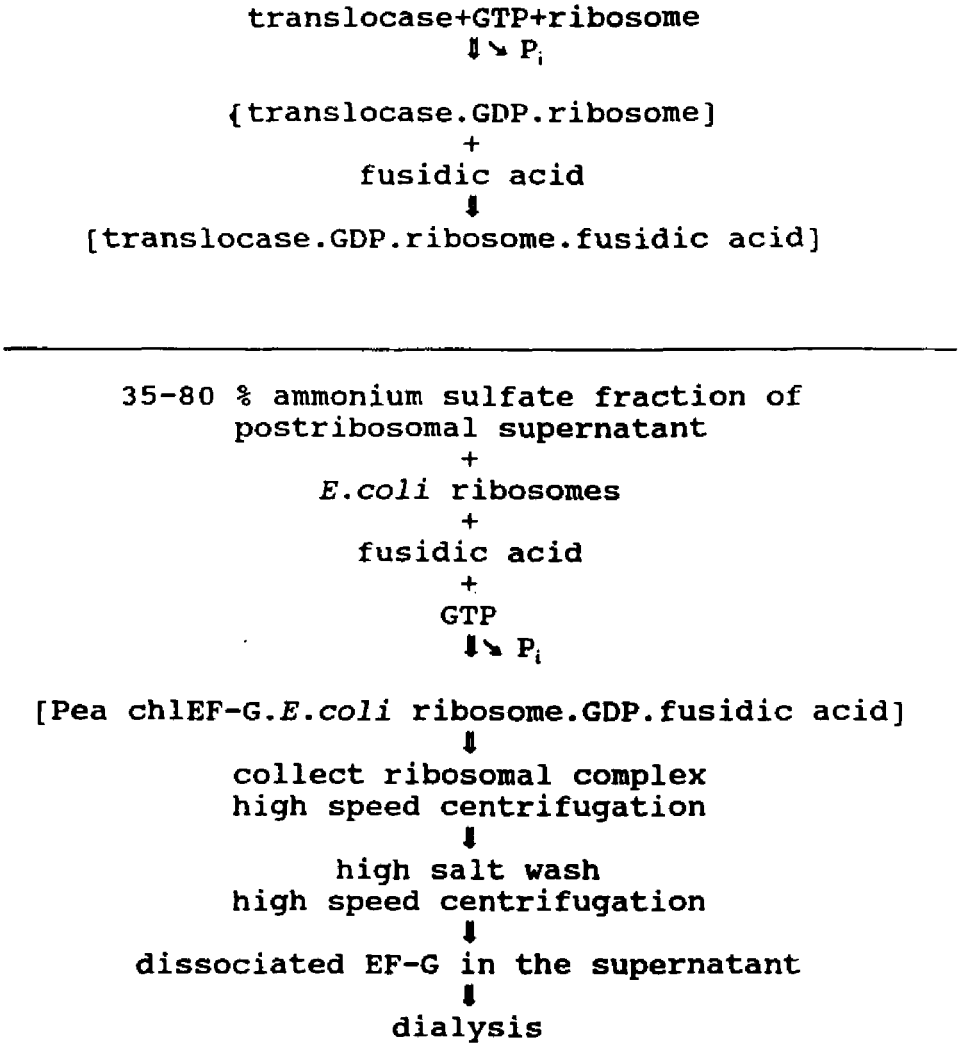


Figure 2: Fusidic acid affinity purification scheme.

translocases on prokaryotic ribosomes which are the ribosomes supplied in the assay mixture. This is critical for the activity measurements of organellar EF-G in crude extracts in which EF-2 is also present.

2.2 Materials and Methods

Preparation of organellar EF-G activity assay components:

Escherichia coli MRE600 were grown in 2YT media (1.6% w/v tryptone, 1.0% w/v yeast extract, 0.5% NaCl, sterilized). 35x1L media were inoculated with 5 mL overnight cultures, grown for 2-3 hrs at 37°C and harvested by centrifugation at 7000 rpm (Beckman JA10 rotor), 4°C before the cells reach the stationary phase. The collected cells were washed in Cell wash buffer (50 mM Tris-HCl, 5 mM KCl, 5 mM $MgCl_2$) by resuspension and centrifugation was repeated, yielding 1.07 g wet cells/L of media. The ribosomes, a complex of elongation factors Tu/Ts, and elongation factor G were prepared from the same extracts.

37.57 g wet weight of frozen cells were disrupted by grinding with 1.5 times cell weight of Alumina-305 in 4°C cold mortar. Grinding was continued for 5 min after the paste was formed. The ribosomes were obtained as described previously (Breitenberger et al., 1979a) by high speed centrifugation, and high-salt wash. They were collected on 20 % sucrose cushion. The concentration of ribosomes was determined by

measuring the absorbance at 260 nm (14.4 A_{260} units/mL is equivalent to 1 mg of ribosomes per mL). The yield of ribosomes was determined to be 11.7 mg/g wet cells.

The first postribosomal supernatant of the ribosomal preparation was fast-frozen and stored at -70°C as a source of transfer factors. EF-Tu and EF-Ts, as a complex (0.67 mg/mL), and EF-G (0.53 mg/mL) were isolated by separation on a DEAE-Sephadex ion exchange column as described in the literature (Ravel et al., 1968)

Crude aminoacyl-tRNA synthetases were prepared from freshly harvested *E. coli* MRE600 cells (~12 g wet cells obtained from 12x1L inoculated media), collected as above. The cells were washed and resuspended in ~40 mL Synthetase buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$) and broken open using a Frenchpress. The supernatant of a 16,000 rpm centrifugation at 4°C for 30 min (Beckman JA10 rotor) was divided into 5 mL aliquots and fast-frozen, and stored at -70°C. The extract was subjected to dialysis against a buffer containing 0.04 M Tris-HCl, pH 7.7, 6 mM ß-ME just before use (Muench et al. 1966), in Spectrapor membrane tubing (MW cut off 12,000-14,000, vol/cm: 0.32mL, from Allied Fisher Scientific).

E. coli MRE600 tRNA (Boehringer Mannheim) was aminoacylated with [^{14}C]Phe (405 mCi/mmol, 236 mCi/mmol, ICN) using a crude extract of aminoacyl-tRNA synthetases prepared as above. A 10 mL reaction mixture contained the following:

10 μCi [^{14}C]Phe, 10 mM $MgCl_2$, 2 mg/mL tRNA (*E. coli* MRE 600), 1.2 mg/mL ATP, 0.1 M Tris-HCl, pH 7.7, 50 ng/mL cold Phe, 100 mM ß-ME ,0.36 mg/mL aminoacyl-tRNA synthetases (the enzyme, tRNA and cold Phe amounts were adjusted to optimum). The reaction mixture was incubated at 37°C for 15 min. The [^{14}C]Phe charged tRNA was phenol extracted and acid precipitated as described previously (Ravel et al. 1971). 439-583 cpm/pmol [^{14}C]Phe-tRNA was obtained depending upon each preparation.

Organellar EF-G activity assay:

This assay measures the translocase activity of EF-G in terms of [^{14}C]Phe incorporation into polyphenylalanine polymers (see Figure 3 for an overview of prokaryotic protein synthesis elongation cycle). 0.1 mL reaction mixture in which poly[^{14}C]phenylalanine is synthesized contains 50 mM Tris-HCl, pH 7.8, 0.1 mM spermidine, 1 mM DTT, 0.37 mM GTP, 5 μg poly(U), 10-12.2 mM $MgCl_2$, 50-72 mM NH_4Cl, 20-50 pmol [^{14}C]Phe-tRNA, 76 μg ribosomes, saturating amounts of partially purified of *E. coli* EF-Tu and EF-Ts complex (5 μL), and varying amounts of crude extracts or fusidic acid purified proteins containing limiting amounts of organellar EF-G or HPLC-purified chloroplast EF-G in limiting levels. After 15 min incubation at 37°C, the reaction was stopped by addition of 10 % trichloroacetic acid (TCA) and heated up to 90°C for 10 min. The ester bond in the aminoacylated tRNA, between

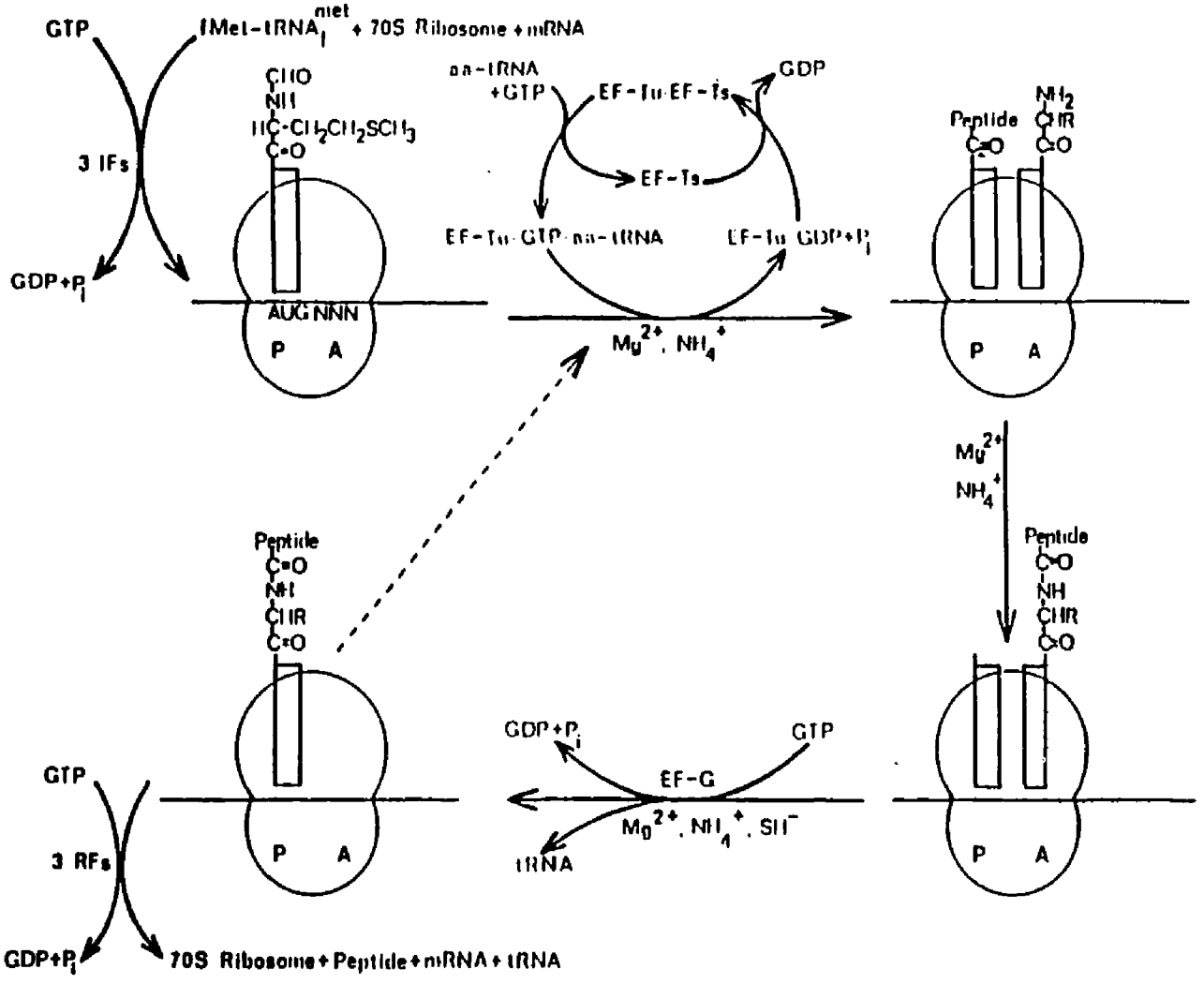


Figure 3: Prokaryotic protein synthesis elongation cycle.

[^{14}C]Phe and tRNA is cleaved at 90°C (Ravel et al., 1971). The precipitated polypeptides were filtered through Millipore type HA, 0.45 μm pore size filters. The filters were then dried and the amount of [^{14}C]Phe quantified by measuring cpm values on the LKB Wallach 1209 Rackbeta Liquid Scintillation Counter. The enzyme specific activity was defined as pmol [^{14}C]Phe incorporated per μg protein (Breitenberger et al., 1979b). Organellar EF-Gs are only functional on prokaryotic ribosomes, while cytoplasmic EF-2, which is also present in the crude extracts, is not (Grivell et al., 1972). Therefore, when supplied with *E. coli* ribosomes this assay only detects organellar (chloroplast and presumably also mitochondrial) EF-G activities.

Growth conditions of pea seedlings:

Pea seeds (*Pisum sativum* var. Progress No.9, Letherman Seed Co., Canton Ohio) were soaked in water one day prior to planting. The day of planting being day zero, usually germination started the day after planting, day one. Plants were watered every other day as required. Purification of chlEF-G was performed from 13 days dark-grown and 3 days light-transferred (11 μmole photons m^{-2} sec^{-1}) pea seedlings. Only the greening uppermost part of the seedlings was harvested from these samples. Partial purification of mitoEF-G was from 13 days dark-grown whole pea seedlings.

Preparation of large quantities of cell extracts:

Peas were grown in the dark for 13 days (-hν) or in the dark for 13 days followed by 3 days under white light (hν↑). Freshly harvested plant tissue (97.7 g of light-induced pea seedlings green tissue, 271 g of dark-grown pea seedlings) was homogenized by using a household blender for approximately 5-10 min blending with 15 sec bursts until the tissue was completely homogenized, in Buffer A (50 mM Tris-HCL, pH 7.8, 50 mM NH_4Cl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, freshly added 6 mM ß-ME and 1 mM PMSF). The homogenate was filtered through two to four layers of cheesecloth. This was followed by two low speed centrifugation steps, i.e. 5000 rpm centrifugation in Beckman JA10 rotor for 10 min at 4°C and the supernatant was collected and subjected to another centrifugation at 15,000 rpm for 20 min at 4°C in Beckman JA20. The postribosomal supernatant was obtained by 55,000 rpm speed ultracentrifugation for 4 hrs, at 4°C in Beckman Ti60 rotor. The top 3/4 of the postribosomal supernatant (PRS) was collected, and a 35-80 % ammonium sulfate fraction (ASF) of PRS of light-induced/dark-grown peas were prepared. PRS was precipitated in 35 % ammonium sulfate and centrifuged. The protein pellet was discarded. To the remaining supernatant, ammonium sulfate was added to make up the final concentration to 80 %, the protein pellet was collected by centrifugation and dissolved in Buffer A. The PRS-ASFs were

then dialyzed against Buffer A (as described previously for the extracts of *E. gracilis*; Breitenberger, 1979a; with some modifications: e.g., the Sephadex G-25 column chromatography step was omitted).

Fusidic acid affinity purification (FAP) of organellar EF-Gs:

The affinity purification of pea chloroplast or mitochondrial EF-G was adapted from the procedure for chloroplast EF-G affinity purification of *E. gracilis* (Breitenberger et al., 1979a). Fusidic acid stabilizes the formation of an EF-G-GDP-ribosome complex. Fusidic acid sodium salt, GTP and *E.coli* ribosomes were added to the postribosomal supernatant of pea extracts in Buffer A (no PMSF) and incubated on ice for 30 min. The mixture was added on 2 mL sucrose cushion (44 % sucrose, 1 mM DTT, 4 mM fusidic acid, 50 mM Tris-HCl, pH 7.8, 10 mM NH_4Cl, 20 mM $Mg(OAc)_2$, 5μM GTP), (adapted from Breitenberger et al., 1980). The ribosomal complex containing EF-G was recovered by high speed centrifugation for 4 hrs, at 55,000 rpm in a Ti70 Beckman rotor using 10.6 mL tubes, at 4°C. This complex was washed by resuspension of pellets in Buffer A (containing 1 mM DTT) overnight at 4°C, in order to eliminate nonspecifically bound proteins to ribosomes, and collected again by centrifugation as above. The EF-G was dissociated from the complex by high-salt wash in 10 mL Buffer B (50 mm Tris-HCl, pH 7.8, 1.0 M

NH_4Cl, 20 mM $Mg(OAc)_2$, 1 mM DTT, 10 % glycerol) and subjected again to centrifugation at 70,000 rpm (150,000 g), for 2.5 hrs in a Beckman TLA-100.3 rotor, at 4°C. The top 3/4 of the supernatant of centrifuged materials were dialyzed against 3 L Buffer A (freshly added 6 mM ßME) for 8 hrs and then against 2.2 L Buffer A for 12 hr at 4°C. Small aliquots were fast-frozen and stored at -70°C.

HPLC purification:

Affinity purified chlEF-G was further purified by size exclusion HPLC utilizing a Waters Protein-Pak 200SW column with dimensions of 8x300 mm. 69.8 µg of FAP EF-G hvt was loaded on Buffer A preequlibrated column with a flow rate of 0.25 mL/min. The separation was followed by UV monitor at 220 nm. Fractions were collected by hand into 1.5 mL microcentrifuge tubes.

Purification of *Euglena gracilis* chloroplast EF-G and immunostaining:

Growth conditions and fusidic acid purification of this protein were as described in (Breitenberger et al. 1980). Electroblotted proteins were immunostained with the protoblot immunostaining kit from Promega.

Protein concentration determination:

Concentrations of proteins in crude extracts were

determined using the BCA assay from Pierce, and bovine serum albumin was used as a standard. Protein concentration determinations of affinity purified EF-Gs were performed according to Hill et al., 1988, to eliminate the interference of ß-mercaptoethanol in Pierce BCA assay system. The concentration of HPLC purified protein was estimated from a silver-stained SDS gel (Morrissey, 1981) by using an automated scanning instrument (USB SciScan 5000). The amount of EF-G estimation was based on a normalization done with a known quantity of PRS-ASFs and bovine serum albumin loaded on the same gel.

2.3 Results

Extraction of peas with different methods of disrupting the plant tissues:

We have tried three different methods of breaking peas; with glass beads (A), by grinding with washed alumina 305 (B), by grinding with acid washed sand (C), and by blending (D). In each case 5 g of frozen light-grown peas (stem and leaves) were used. The postribosomal supernatants were prepared as in Materials and Methods in Chapter II and stored at -70°C. The extracts were assayed for EF-G activity. Table 1 displays the results obtained.

Table 1: Comparison of various methods for releasing organellar EF-G from pea seedlings.

Method	% relative specific activity
A	28
B	19
C	100
D	63

Concentration determination and activity assay as in Materials and Methods of Chapter II. The highest units/μg (0.120 units/μg) of extract was considered to be 100 %.

The best way of releasing organellar EF-G from pea seedlings pea was found to be grinding with sand among these tested methods (specific activity is 0.120 units/μg). However we obtained pea extractions for large scale preparations by using a blender (with best estimation, "blender" method results in about 37 % less specific activity), since above methods would not be very practical.

Preparation of ammonium sulfate fractionated extracts:

The very first step in the purification was to eliminate the cellular ribosomes from the whole cell extracts, otherwise cellular ribosomes would contaminate the affinity purification step and interfere in the protein synthesis assay. Postribosomal extracts were concentrated by ammonium sulfate fractionation. Figure 4A, lane 1 and Figure 4B, lane 1 are ammonium sulfate fractionated postribosomal extracts from light-induced and dark-grown pea, respectively. Table 2 compares the EF-G specific activities of materials obtained at

every step of the purification. The yield at the very first step of the purification process was designated to be 100 %. When the EF-G activity of the ammonium sulfate fractionated postribosomal supernatants of light-induced and dark-grown peas were compared, a 3.8-fold increase was observed in those exposed to light. The comparisons with crude extracts (Chapter III, result section) showed at least 10-14 fold of chlEF-G specific activity difference between light and dark grown peas for 10-13 days. Also three days light-induced 13 days dark adapted peas resulted in 2.3-fold chlEF-G specific activity increase compared to 13 days old continuous white light grown peas. We would expect a 25-fold activity difference between dark-grown and light-induced peas, based on the values above. This discrepancy in the results is probably due to a leakage of some light during the dark-growth period for this specific sample preparation. This would also explain why a band corresponding to 86,000 molecular weight is present on the gel shown in Figure 4B, lane 2; which is absent in Figure 13; lanes 7, 8 and 9.

Fusidic acid affinity purification:

EF-G was further purified from ammonium sulfate fractionated extracts of both dark-grown and light-induced peas by fusidic acid affinity purification to compare the EF-G specific activity in each case (Figure 4B, lane 2 and Figure 4A, lane 2; respectively). Figure 4A lane 2 is a control for

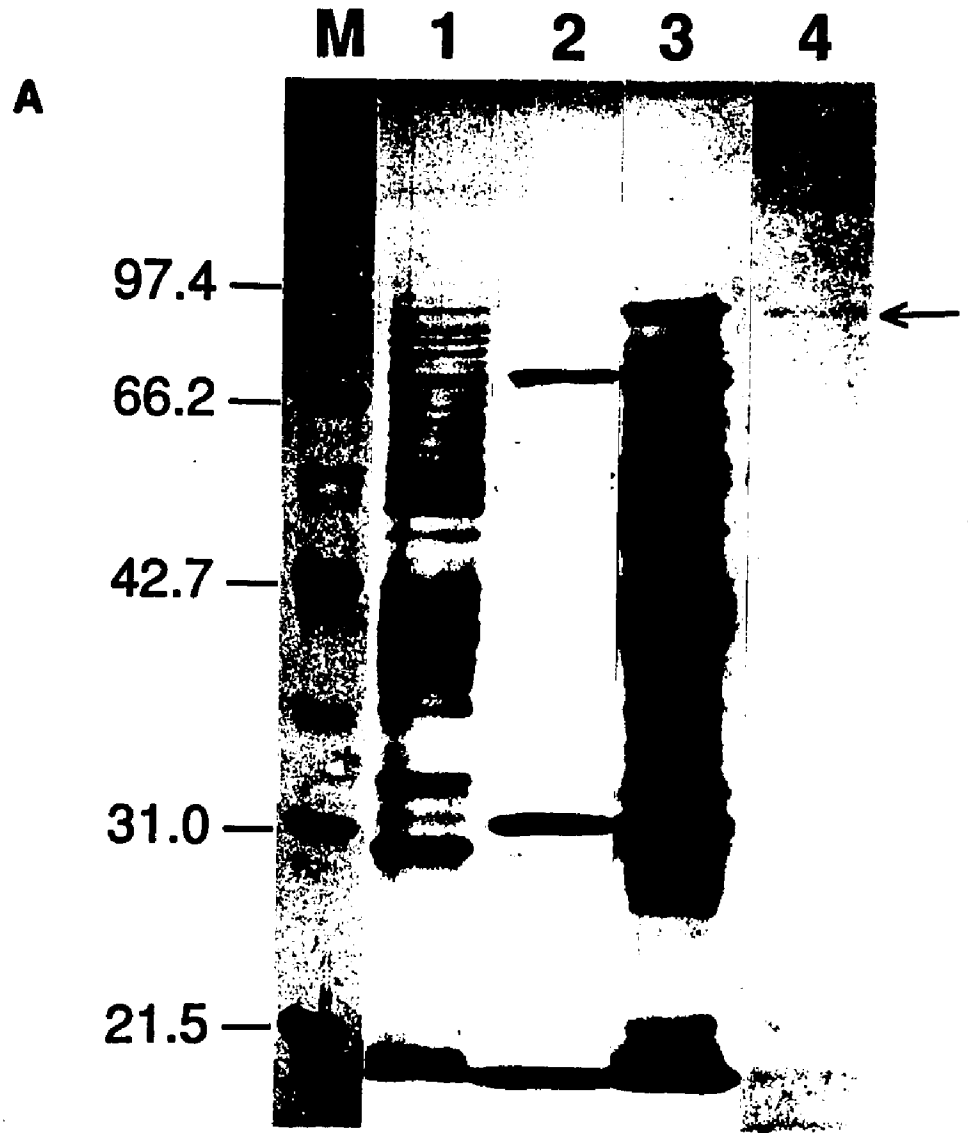


Figure 4: SDS-gel electrophoresis of proteins at different purification levels. **A.** Samples from purification steps indicated in Table 1 were subjected to SDS-PAGE (8% gel) and silver stained. Lanes: M, molecular weight markers, 1. PRS-ASF hν↑ (3μg), 2. FAP control (1.8μg) in which the fusidic acid affinity procedure was carried out without the addition of pea extract. This control shows that some proteins are obtained from the ribosomes alone., 3. FAP EF-G hν↑ (5μg), 4. HPLC purified chlEF-G (61.7ng).

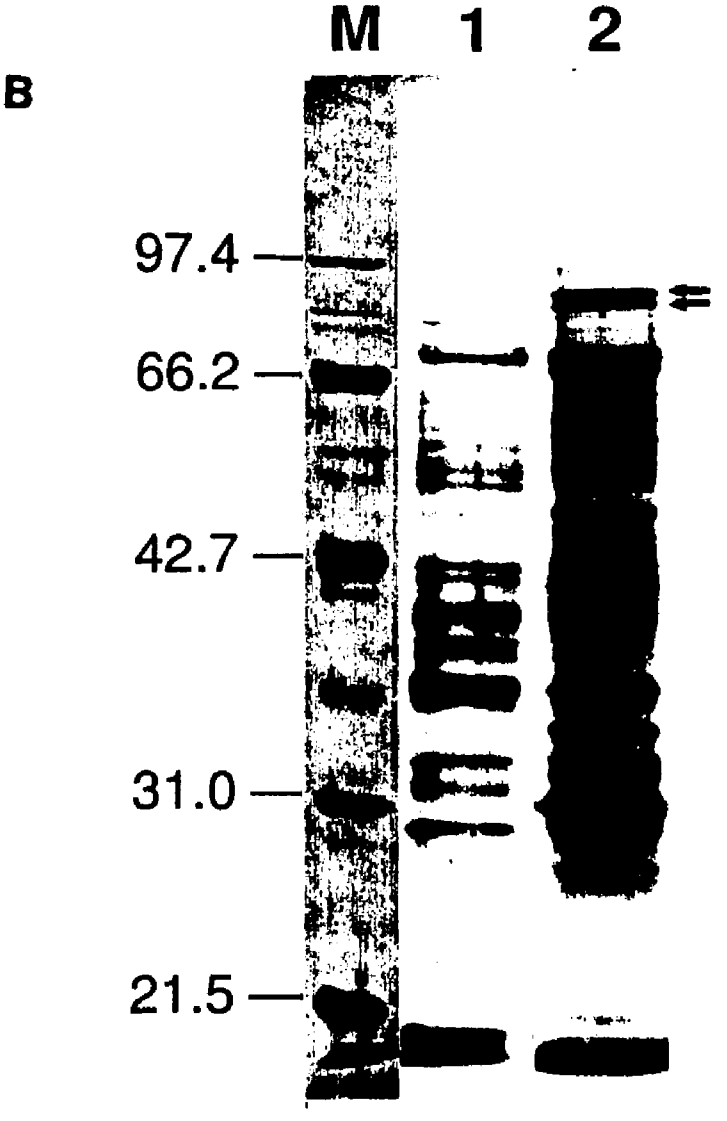


Figure 4 (continued) B. M, molecular weight markers, 1. PRS-ASF -hν (3μg), 2. FAP EF-G -hν (8.9 μg).

Table 2: Purification of chloroplast EF-G.

Fraction:	Protein (mg)	Units (pmol phe)	Specific Activity (pmol/μg)	Purification (-fold)	Yield (%)
PRS-ASF hν↑	197.5	96775	0.49	1	100
FAP EF-G hν↑	2.72	38352	14.1	28.8	39.6
HPLC-chlEF-G hν↑	6.30×10^{-4}	451	716.2	1,462	0.5
PRS-ASF -hν	195.5	25415	0.13	1	100
FAP EF-G -hν	2.83	2971.5	1.05	8.07	11.7

Magnitude of purification and percent yield values at every step were obtained from calculations where it was assumed that all the sample was used from the previous step for the next step.

the fusidic acid affinity purification step; in the absence of added crude extract, the ribosomal pellet releases two proteins of different molecular weights than that of chlEF-G.

The yield and degree of purification was relatively low in the case of dark-grown material. At the end of this purification step fusidic acid was removed by dialysis against Buffer A. Although dialysis conditions (time, volume) were the same based on the amount of protein dialyzed, the extent of fusidic acid removal may be somewhat different. We know from the later steps that the EF-G specific activity of light-induced material is the correct value (residual fusidic acid does not mask the true activity), since the "ideal" EF-G specific activity is very close to the activity obtained from the HPLC-purified protein (Table 7). We cannot do a similar analysis for the dark-grown material due to the lack of data, we do not have the specific activity of mitoEF-G at the HPLC-purification step. Nevertheless, with these comparisons, the light induction observed in studies with only crude extract in Chapter III can be confirmed.

The affinity purification resulted in partial purification of proteins from both dark-grown and light-induced peas (Figure 4). Therefore, HPLC purification of chlEF-G was pursued to determine if the activity observed with fusidic acid material is due to the protein band seen on SDS gels corresponding to a molecular weight of 86,000, (Figure 4A, lane 2) which is very similar to the molecular weight of

85,000 for *E. gracilis* chlEF-G (Figure 5). We did not obtain a clearly resolved peak of chlEF-G since the protein was eluting very close to the void volume of the column used (Figure 6). Then, we did not attempt to separate the protein that is presumed to be mitochondrial EF-G which has a very close molecular weight to its chloroplast counterpart (see Figure 4B for mitochondrial EF-G, see Figure 6 for the elution profile).

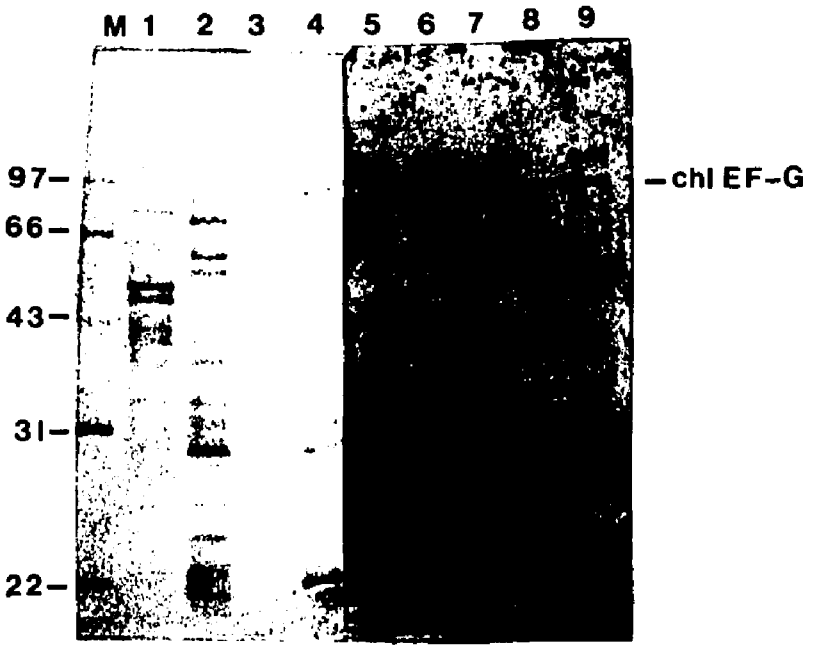


Figure 5: Comparison of pea and *E. gracilis* chloroplast EF-G molecular weights. The panel on the left is the Coomassie blue stained PVDF-membrane. The right panel is immunostained PVDF-membrane with antibodies against *E. gracilis* chlEF-G. The same amounts of materials were loaded to the gels for each panel. Lanes; M. molecular weight markers, 1. Pea PRS-ASF, 2. *E. coli* ribosomal proteins, 3. *E. gracilis* chlEF-G, 4. Pea chlEF-G ribosomal complex, 5. *E. gracilis* PRS-ASF, 6. *E. coli* ribosomal proteins, 7. FAP control, 8. FAP *E. gracilis* chlEF-G, 9. *E. gracilis* chlEF-G ribosomal complex

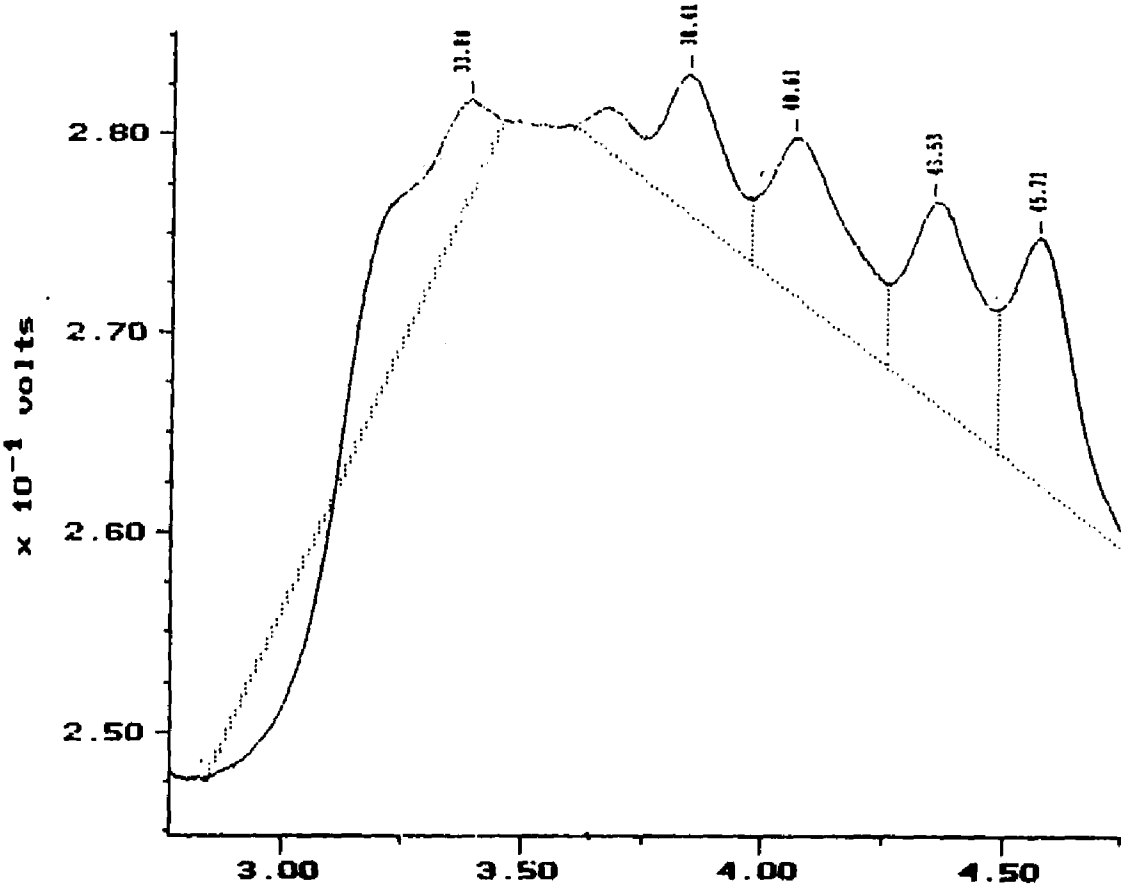


Figure 6: HPLC elution profile. The fractions corresponding to the first peak (not well resolved, shoulder of "33.88") and other peaks were collected separately.

Further removal of fusidic acid from the affinity purified material:

Gel filtration column chromatography was performed with both dark grown and light-induced extracts on the same column under the same conditions. 800 μL protein was loaded on 8 mL Sephadex G-100 column equilibrated with Buffer A overnight at 4°C. 250 μl fractions (102 sec/fraction) were collected and assayed for EF-G activity. Table 3 shows the data obtained.

Total activity (in units) recovered from the FAP hν↑ column (fractions 10-17) eluted is 1672.1 units. 800 μL of 2.3668 units/μL FAP hν↑= 1893.5 units was loaded. 88.3 % of the EF-G activity loaded on the column was recovered. However total units recovered from the FAP -hν column (fractions 12-17) is 333.5 units. 800 μL of 0.268 units/μL FAP -hν=214.6 units was loaded. The recovery is 155 %, representing 1.5-fold increase in units of EF-G. This apparent increase may simply represent the removal of fusidic acid or some other low molecular weight impurity.

Table 3: Sephadex G100 gel filtration: Collected fractions with EF-G activity.

Sample	Fraction #	amount assayed(μL)	cpm([^{14}C]Phe)
FAP hν↑	10	14	1374.22
	11	"	6125.27
	12	"	9022.45
	13	"	8980.56
	14	"	8195.52
	15	"	5772.03
	16	"	5063.70
	17	"	2941.51
FAP -hν	12	30	1762.94
	13	"	4118.55
	14	"	4330.14
	15	"	4479.25
	16	"	3650.17
	17	"	1949.13

HPLC purification:

The quantity of chlEF-G obtained from HPLC purification was estimated by scanning of a silver-stained gel containing a known volume of HPLC-purified protein (Materials and Methods). Scanning of SDS gels like those shown in Figure 4 on USB SciScan 5000 instrument allowed us to estimate the ideal maximal specific activity of chlEF-G. The peak areas from the scanned gel were calculated in arbitrary intensity units (AIU) as given in Table 4.

Table 4: Arbitrary intensity units (AIU) of total protein per lane or amount of protein in a single band.

Samples	Proteins	AIU
a	FAP -hν (lane 2, Figure 4B)	24.70
b	FAP +hν (not shown)	16.24
c	FAP hν↑ (lane 3, Figure 4A)	19.08
d	PRS-ASF -hν (lane 1, Figure 4B)	12.76
e	PRS-ASF +hν (not shown)	11.58
f	PRS-ASF hν↑ (lane 1, Figure 4A)	10.35
g	chlEF-G, 86kD band (lane 2, Figure 4B)	0.25
h	mitoEF-G, band below 86kD (lane 2, Figure 4B)	0.30
i	chlEF-G, 86kD band (of sample b)	0.31
j	chlEF-G, 86kD band (lane 3, Figure 4A)	0.34

To approximate the amount of protein present, the total AIUs for the three lanes (samples; d,e,f) containing PRS-ASFs were averaged, and divided by the amount of protein loaded (3 μg per lane, based on BCA assay). This yielded a ratio of 3.81 AIU/μg of protein. This value was then used to estimate the total protein in each lane as well as the amount of protein in the band corresponding to EF-G (Table 5).

The percent error range is about 30 %, estimated from the AIU value of a known quantity of Bovine serum albumin loaded on the very same gel.

Table 5: The calculated amounts of samples in Table 3.

Samples	calculated amounts (μg)
a	6.480
b	4.260
c	5.010
d	3.350
e	3.040
f	2.720
g	0.066
h	0.079
i	0.081
j	0.089

The relative intensity of HPLC-purified chlEF-G on the gel was 18.15 % of EF-G band of FAP hvt calculated from AIU. So; 0.34 AIU/ (FAP chlEF-G hvt) x18.15 % (chlEF-G/FAP chlEF-G hvt)x μg/3.81 AIU=0.016 μg chlEF-G present in the gel. HPLC-purified chlEF-G was loaded after 8-fold concentration (concentrated by filtration on Millipore Ultrafree MC tubes with a low binding type PLGC ultrafiltration membrane, for 1 hr 5,000 g centrifugation) = 200 μL unconcentrated fusidic acid affinity purified material. This resulted in concentration determination of 8.1×10^{-5} mg/mL HPLC-purified chlEF-G. From direct activity assay it was estimated that HPLC-purified chlEF-G has an activity of 0.058 units/μL. Since the concentration could be estimated, now the specific activity can be calculated as (0.058 units/μL) / (8.1×10^{-5} μg/μL)= 716.2 units/μg. This value is very close to the "ideal" specific activity of chlEF-G calculated by measuring the intensity of the 86kD band in FAP chlEF-G hvt (Table 7).

We also used these values to estimate the maximal specific activity of EF-G as mentioned above, assuming that the uppermost chlEF-G band accounts for all the EF-G activity. Combining the values of specific activities in Table 2 and % EF-G values in Table 6 it is possible to estimate the maximal specific activity of chloroplast and mitochondrial EF-Gs, and are listed in Table 7.

Table 6: The percentage of chloroplast or presumable mitochondrial EF-G in fusidic acid purified materials.

Protein	% EF-G in total FAP
EF-G -hν in a; lower band (mitoEF-G)	1.22
EF-G -hν in a; upper band (chlEF-G)	1.01
EF-G +hν (not shown) chlEF-G	1.91
EF-G hν↑ in c; chlEF-G	1.78

Table 7: "Ideal" EF-G specific activities.

Sample	EF-G specific activity pmol/μg
EF-G upper band (chl)in c (Fig.4A, lane 3)	794.0
EF-G upper band (chl)in a (Fig.4B, lane 2)	86.06
EF-G in HPLC peak1 (Fig.4A, lane 4)	716.2
EF-G in HPLC peak2 (not shown)	600.0

The take home lesson is that the values for chlEF-G specific activity are very similar in the proteins purified from light-grown pea seedlings and dark-adapted light-induced pea seedlings. However, chlEF-G in the FAP -hν sample is not

as active. There may be at least a 9.2-fold specific activity difference between the EF-Gs obtained from light-induced and dark-grown peas (compare 794 units/μg with 86.06 units/μg). The activity difference of 9.2 is comparable to the difference obtained when fusidic acid purified proteins are compared, 13.4-fold (Table 2). There may be something inhibitory in the -hv extract. This could explain the 1.5-fold activity increase obtained from Sephadex G-100 size exclusion chromatographed FAP -hv material. Also the lower band assumed to be mitoEF-G is not as active on *E. coli* ribosomes either. Since the main focus of this work is on light-induced chlEF-G we did not pursue this observation.

After all, scanning allowed us to compare purification steps. In conclusion, a 1,462-fold purification was obtained from crude extracts to HPLC-purified material. The "ideal" EF-G specific activity of fusidic acid purified material and the specific activity of HPLC-purified material were very similar, indicating that there was little fusidic acid contamination in the light induced sample.

Molecular weight comparison of *Euglena gracilis* pea chlEF-G:

Chloroplast EF-G from *Euglena gracilis* was purified as described in Breitenberger et al., 1980 in order to compare its known molecular weight to the molecular weight of pea chlEF-G. As shown in Figure 5, the molecular weights are

close; pea chlEF-G was found to be around 86,000 D. This comparison let us assume that the major band at that relative mobility to be chlEF-G, which was proven to be correct by HPLC purification.

Sensitivities of pea chlEF-G and *Euglena gracilis* chlEF-G to fusidic acid:

The fusidic acid sensitivity of translocases is a general phenomenon. However the amount of fusidic acid required to obtain the same degree of inhibition of EF-G activity differs among the different organellar EF-Gs (chloroplast and mitochondrial) and different species; high levels of resistance was observed with mitoEF-G to fusidic acid, chlEF-Gs were more sensitive to this antibiotic (Breitenberger et al., 1980). The results suggest structural differences at sites involved in forming the ribosome-bound complexes, in addition to differences in catalytic activity. Hence, we compared the sensitivities of pea chlEF-G and *E. gracilis* chlEF-G to fusidic acid (Figure 7).

Although, pea chlEF-G showed essentially same sensitivity to fusidic acid as *E. gracilis* chlEF-G, the antisera against *E. gracilis* chlEF-G did not cross-react with pea chlEF-G (data not shown).

Figure 7: Sensitivities of pea chlEF-G and *E. gracilis* chlEF-G to fusidic acid. To the ammonium sulfate fractionated postribosomal supernatants (PRS-ASF) (8.55 μg pea PRS-ASF, 0.84 μg *E. gracilis* PRS-ASF) fusidic acid was added in indicated final concentrations to 100 μL assay mixture, then the samples were assayed for decrease in EF-G activity. The change in the activity was illustrated as % relative activity, considering the initial activity 100 % (with no fusidic acid, initial activity for pea is 0.42 units/μg, for *E. gracilis* is 3.25 units/μg).

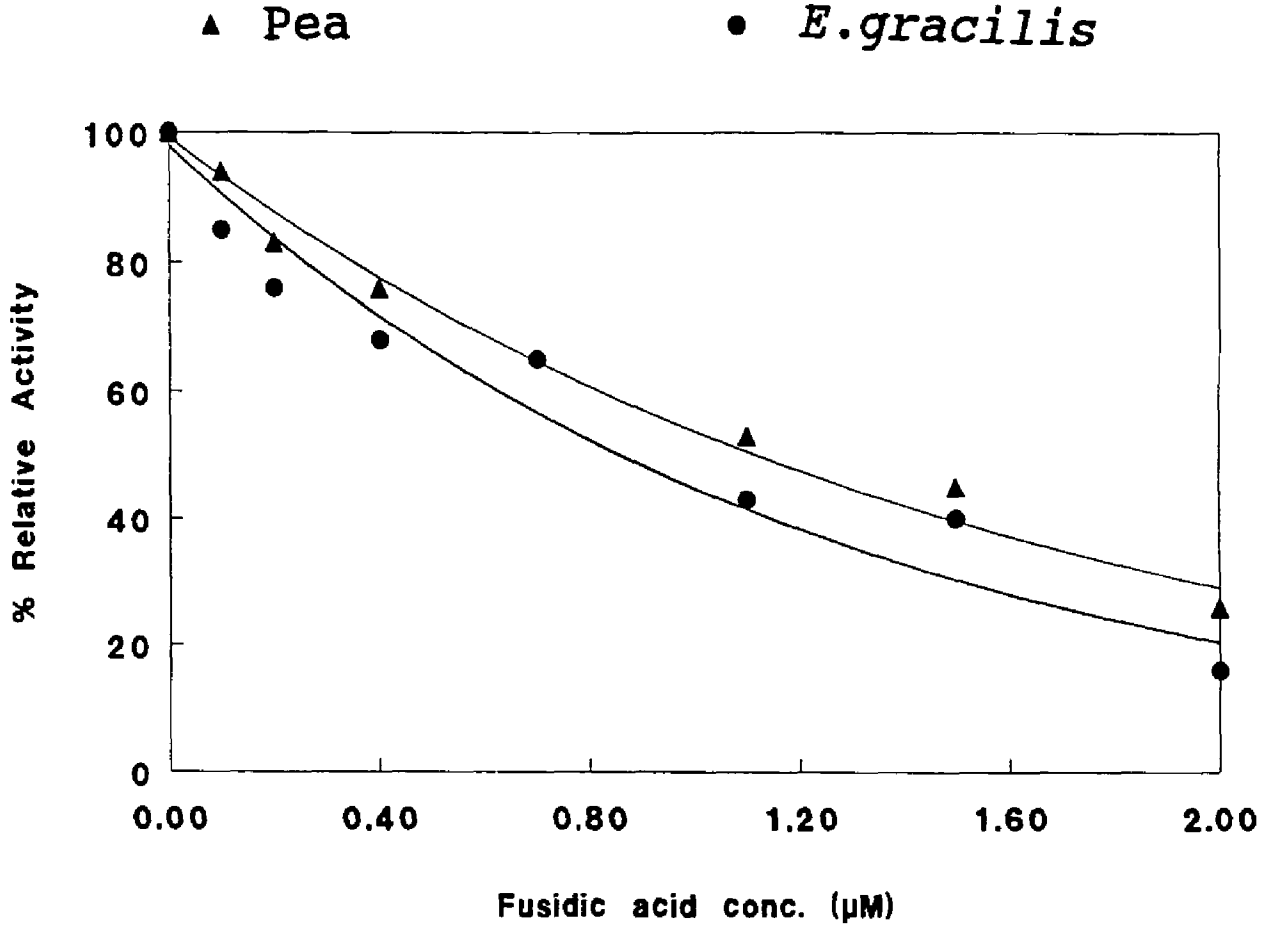

▲ Pea
● E.gracilis
100
80
60
40
20
0
% Relative Activity
0.00
0.40
0.80
1.20
1.60
2.00
Fusidic acid conc. (μM)

Figure 7

2.3 Discussion

Comparisons of the EF-G activity in partially purified preparations from dark-grown and light-induced pea seedlings suggested a 4 to 14-fold activity increase in the light-induced samples. The relevance of the magnitude of this induction is discussed in chapters 3, 4 and 5.

In order to obtain the highly purified material from HPLC (shown in Figure 4A, lane 4) we collected only the leading edge of the EF-G peak. This resulted in a low yield (0.5 %) of EF-G active band by HPLC. The bulk of the EF-G activity coeluted with an overlapping peak containing other proteins (second peak in the HPLC elution profile (peak 2), data not shown).

HPLC purification was very important in confirming the identity of chlEF-G. A sample was prepared for N-terminal amino acid sequencing from a recomplexed partially affinity purified protein (see Materials and Methods in Chapter IV), however it was expected to be chlEF-G due to the similarity in molecular weight to *E. gracilis* chlEF-G. The result of HPLC purification demonstrated that the band cut out of the membrane (Materials and Methods, Chapter IV) was correctly attributed to the chlEF-G, since the HPLC purified protein comigrated, having chlEF-G activity, at the same molecular weight on the SDS-gel as the sample prepared for amino acid microsequencing.

Purification of spinach chlEF-G from isolated chloroplast following steps of ammonium sulfate fractionation, Sephadex G-100, DEAE-cellulose, a second Sephadex G-100, Hydroxyapatite column chromatographies resulted in 136-fold of purification with 2.1 % yield (Tiboni et al., 1978). Our purification of pea chlEF-G yielded 1,462-fold purification with 0.5 % yield. The affinity purification of *E. coli* EF-G, by the same affinity method (Rohrbach et al., 1974) that we have used, gave 56 % yield , with 25-fold purification after affinity purification step. We have obtained 38.8 % yield with 28.8-fold of purification after the same step.

CHAPTER III

LIGHT INDUCTION OF PEA CHLOROPLAST EF-G

3.1 Introduction

The chlEF-G specific activity studies at different light conditions and time points were performed on whole cell extracts as opposed to isolated chloroplast extracts because of the low yield and variability of chloroplast isolation which would not allow accurate comparisons among the time points. There are, however, presumably three translocases in the whole cell extracts; chloroplast EF-G, mitochondrial EF-G, and cytoplasmic EF-2 (cytoplasmic counterpart of EF-G). Cytoplasmic EF-2 as an eukaryotic elongation factor is not functional on prokaryotic ribosomes (Grivell et al., 1972) and our assay utilized *E. coli* ribosomes. Mitochondrial EF-G is present in very low quantities in the whole cell extracts of light-grown peas. Therefore, most of the activity measured in the whole cell extracts of light grown peas is probably due to chlEF-G.

3.2 Materials and Methods

Growth conditions of peas:

For germination and watering see Materials and Methods section of Chapter II. Peas were grown at different light conditions: White light of fluorescent light bulbs, "Warm White" General Electric, the light intensity, measured by LI-185B quantum photometer ((LI-COR, Inc., Lincon, NE), at the surface of the vermiculite was 11 μmol photons m^{-2} sec^{-1} between 300-850 nm (3.8μmol photons m^{-2} sec^{-1} of total intensity is red light). Red light in 600-850 nm range was obtained by filtering white light through red plexiglass (Rohm and Haas) and the flux was adjusted to 4.0 μmol photons m^{-2} sec^{-1} on the surface of the vermiculite by laying 2 layers of cheesecloth on the plexiglass. Dark-grown peas were grown in tightly sealed boxes in a dark room. The time points of development will be indicated in figures. In the transfer experiments, light-grown peas were transferred to dark, dark-grown peas were transferred to light.

Preparation of whole cell extracts:

Two to five g plant tissue (stems and leaves) were homogenized in 5-10 mL Buffer A using a Tekmar Tissuemizer until tissue was completely homogenized. After removal of cell debris by low speed centrifugation at 5000 rpm in Beckman

JA20 rotor, for 5 min at 4°C, postribosomal supernatants were obtained by high speed centrifugation for 2.5 hrs at 70,000 rpm in a Beckman TLA-100.3 centrifuge, at 4°C (Breitenberger et al., 1990). The concentrations of whole cell extracts ranged from 3-11mg/mL as determined by using the Pierce BCA protein concentration determination assay kit.

Preparation of ribosomal complexes:

The postribosomal supernatants of peas were prepared as above. Ribosomal complexes of extracts from light- and dark-grown peas at different day of development were prepared. EF-G was complexed with ribosomes in the presence of fusidic acid, and GTP. Complexing mixtures (2.5 mL) contained 6 mg of postribosomal supernatant in Buffer A (50 mM Tris-HCl, pH 7.8, 50 mM NH_4Cl, 0.1 mM EDTA, 5 mM $MgCl_2$, 10 % glycerol) 1.5 mg of *E. coli* ribosomes (ratio of protein / ribosome (mg/mg) is 4), 20 μM GTP, 10 mM fusidic acid, 1 mM DTT. Mg^{2+} concentration was brought up to 20 mM by adding 1 M $Mg(OAc)_2$. This mixture was layered on 0.6 mL of sucrose cushion (Materials and Methods, Chapter II), in 3.5 mL centrifuge tubes. After centrifugation for 2.5 hrs, at 4°C, at a speed of 70,000 rpm (150,000xg) in a Beckman TLA 100.3 centrifuge, the ribosomal complexes were suspended in 100 μL Buffer A containing 1 mM DTT. Aliquots of these samples were subjected to electrophoresis on an SDS-Gel (Figure 12).

Isolation of chloroplasts and preparation of crude extracts:

Chloroplast preparation was performed from freshly harvested 13 day old light grown peas (34.76 g stem and leaves) homogenized in a household blender for 3-5 min with 5 sec bursts in 140 mL isolation buffer containing 0.35 M sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, 2.5 % Ficoll 400 (Sigma), 50 mM Tris-Cl, pH 7.6, 1 mM ß-ME, 0.1 % Bovine serum albumin (Sigma). The homogenate was filtered through 4 layers of cheesecloth and 1 layer of Miracloth (Calbiochem), followed by centrifugation for 10 min at 75xg, 4°C. The supernatant was subjected to another centrifugation for 15 min, at 1,020xg, 4°C. 1.63 g wet weight of chloroplast pellet was obtained (Palmer, J. D. 1986) and resuspended in 13 mL Buffer A containing 1 mM DTT instead of ß-ME. 1/3 of this suspension was homogenized (Tekmar Tissuemizer) and sonicated for 10 min with 5 sec bursts and the postribosomal supernatants were obtained as above except centrifugation was for 2 hrs. The concentration of chloroplast extract was 2.0 mg/mL.

Assays:

Activity assays were performed as explained in Methods and Materials section of Chapter II, as well as the protein concentration determination assay of whole cell extracts and extracts of isolated chloroplasts. The EF-G specific activity

at time points indicated in Figure 9 was obtained with at least three increasing amounts of whole cell extracts but subsaturating amounts of chlEF-G, which give a linear increase of activity. The slope of these three points was then plotted as relative specific activity versus day of development. In Figure 8, the assay was repeated three times with the same samples for every time point (excluding the eighth day of development). The average slope obtained with three subsaturating amounts of chlEF-G at each time point was plotted versus day of development. Deviations from the average values were indicated with error bars.

Quantitation of Rubisco subunits in whole cell extracts:

The same amounts of whole cell extracts of light-grown, dark-grown, light to dark and dark to light transferred peas at different day of development were subjected to SDS gel electrophoresis and stained with Coomassie Brilliant Blue R-250. The intensities of bands corresponding to LSU at M_r 55,000 and SSU at M_r 14,000 were measured by scanning densitometry with a Hoefer densitometer and plotted. The relative intensities were determined by weighing the cut out photocopies of LSU, SSU peaks of the chromatograms.

3.3 Results

Comparison of chlEF-G activity of the isolated chloroplast and whole cell extracts:

To show that pea chlEF-G is active on *E. coli* ribosomes, chlEF-G specific activity of an isolated chloroplast extract was compared to the chlEF-G specific activity of a whole cell extract. It was expected to obtain increased chloroplast EF-G specific activity from an extract of isolated chloroplasts. In Table 8, comparison of EF-G activity in whole cell and chloroplast extracts is presented. There is 2.13-fold enrichment of EF-G specific activity in the chloroplast extract when compared to the whole cell extract of peas grown for the same number of days, under the same light conditions (Table 8). This experiment is important to prove that the pea chlEF-G can substitute for *E. coli* EF-G in our assay system on *E. coli* ribosomes. Hence, a significant proportion of the EF-G activity detected in extracts from pea seedlings corresponds to the chloroplast factor, chlEF-G.

Table 8: Comparison of EF-G activity in whole cell and chloroplast extracts.

Extract:	Protein: (mg)	EF-G Activity: (pmol[^{14}C]-Phe)	Specific Activity: (units/μg)
Chloroplast	8.6	1376	0.16
Total/light	184	13821	0.075
Total/dark	103	165	0.0016

Postribosomal supernatant was prepared from isolated chloroplasts from 13 day light-grown seedlings or from seedlings grown in the light or in the dark for 13 days (Total/light and Total/dark, respectively). The preparations of postribosomal supernatants, concentration determinations of total proteins, and activity measurements were as explained in Materials and Methods. The values given in the table are normalized to correspond to the same amount of starting plant tissue (11.6 g wet weight) in each case.

ChlEF-G specific activity in the light grown peas:

The specific activity of EF-G is low for the first six days after sowing (Figure 8). Between days 6 and 8, EF-G specific activity increases 7-fold and reaches a maximum after 8 days. The activity then remains stable through day 15. Later, EF-G activity starts to decline, probably due to the maturation of chloroplasts (Woolhouse, 1984).

In light-grown peas; three different points were assayed between 10-40 μg of crude extracts for three times, and the average slope from these three points were plotted in Figure 8. EF-G activity either was not observed or it was at the lower limits of detection in equivalent amounts of extracts from seedlings grown in total darkness. However, assays with 50 μg dark-grown pea crude extracts resulted in

Figure 8: Light induction of chloroplast EF-G. Peas were grown under continuous light (11 μmol photons m^{-2} sec^{-1}) at the indicated time points. Average specific activity (pmol [^{14}C]-Phe incorporated per μg of protein) of each sample, with protein concentrations of 3-11 mg/mL, is expressed as a percentage of the specific activity of the sample prepared from 13 day white light grown peas (0.075 units/μg).

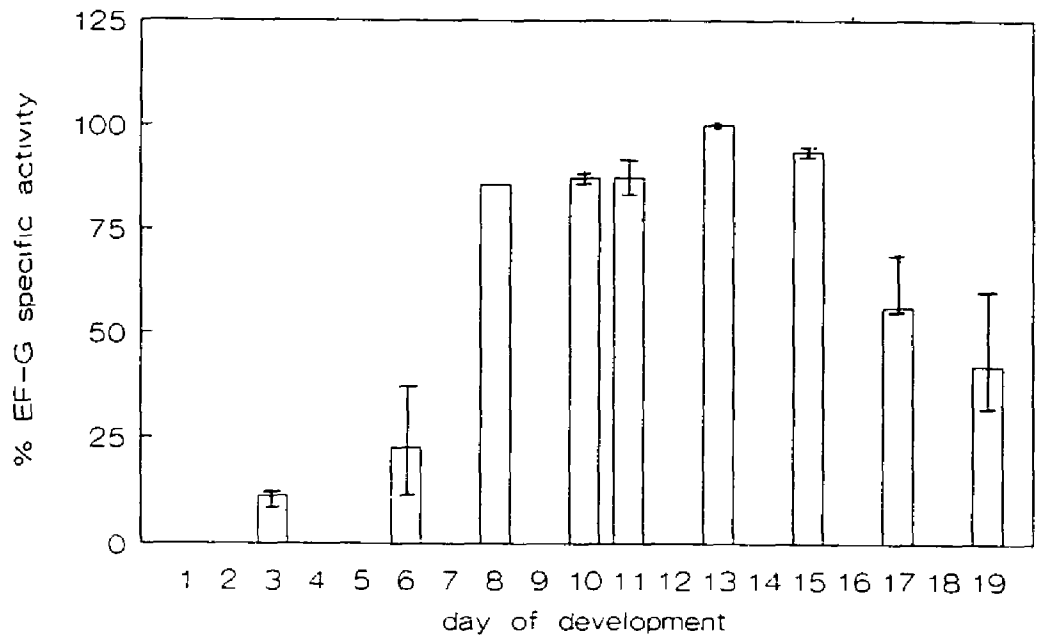

% EF-G specific activity
125
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
day of development

Figure 8

measurable EF-G activities. Considering the EF-G specific activity of 13 day old sample as 100 %, the relative EF-G specific activities of dark-grown pea extracts, based on the single measurement using 50 μg of crude extract are, 3.6 % for 3 day; 10 % for 10 day; 12 % for 11 day; 7.3 % for 13 day; 10 % for 15 day old samples. There is a 13.4-fold difference between the dark- and light-grown 13 day old peas in EF-G specific activity. These data indicate that light is required for maximal activity of chlEF-G.

The red light effect on chlEF-G specific activity:

Whole cell extracts of peas grown under continuous red light for 4,6,8 and 13 days (exposed to the same red light intensity present in white light) were assayed for chlEF-G specific activity (Figure 9). The specific EF-G activity of each sample was expressed as a percentage of the specific activity of the sample prepared from 13 day white light grown peas (0.075 units/μg). Chloroplast EF-G activity of samples prepared from 4, 6, 8 days red light grown peas exhibited increasing EF-G specific activity and a similar maximum to that observed with the white light grown peas by day 8. However EF-G activity was lower on day 13 indicating a different time course of chlEF-G activity under red light (Figure 9). Since the red light-grown seedlings were more elongated and less green than the seedlings of white light-grown peas (Figure 10), a different time course of chlEF-G

Figure 9: The effect of red light on chloroplast EF-G activity. Peas were grown under continuous white light (open bars, 11 μmol m^{-2} sec^{-1} photons) and red plexiglass filtered light (slashed bars, exposed to the same amount of red light, i.e. 600-850 nm) at the indicated time points. Average specific activity (pmol [^{14}C]-Phe incorporated per μg of protein) of whole cell extracts of these samples is expressed as a percentage of the specific activity of the sample prepared from 13 day white light grown peas (0.075 units/μg).

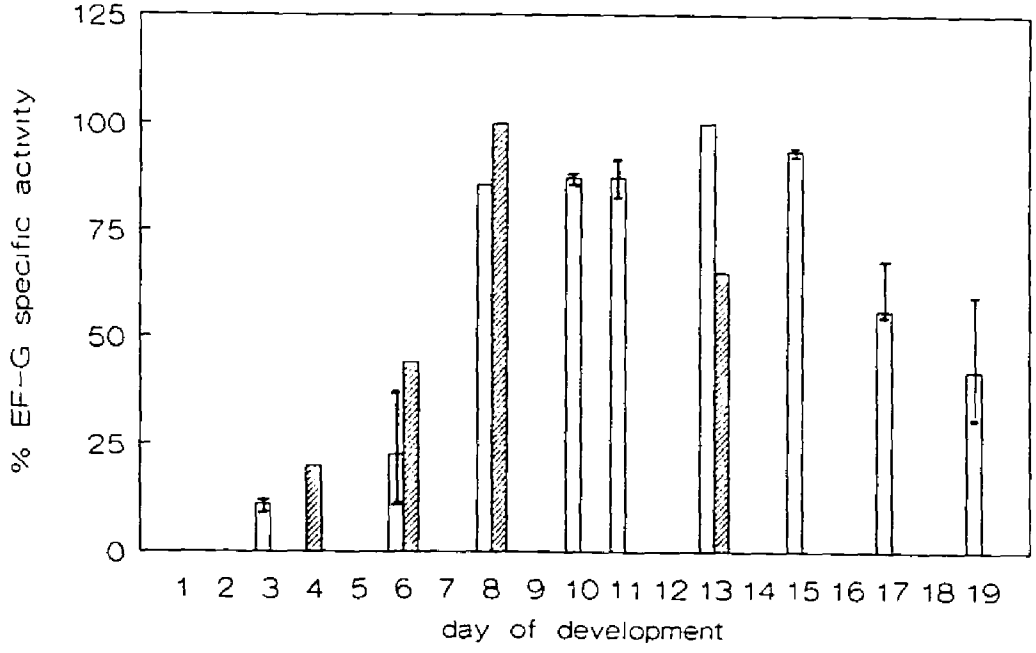

125
100
75
50
25
0
% EF-G specific activity
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
day of development

Figure 9

induction is not unexpected. Nevertheless red light alone was sufficient to induce EF-G activity, suggesting that its induction may be phytochrome-mediated.

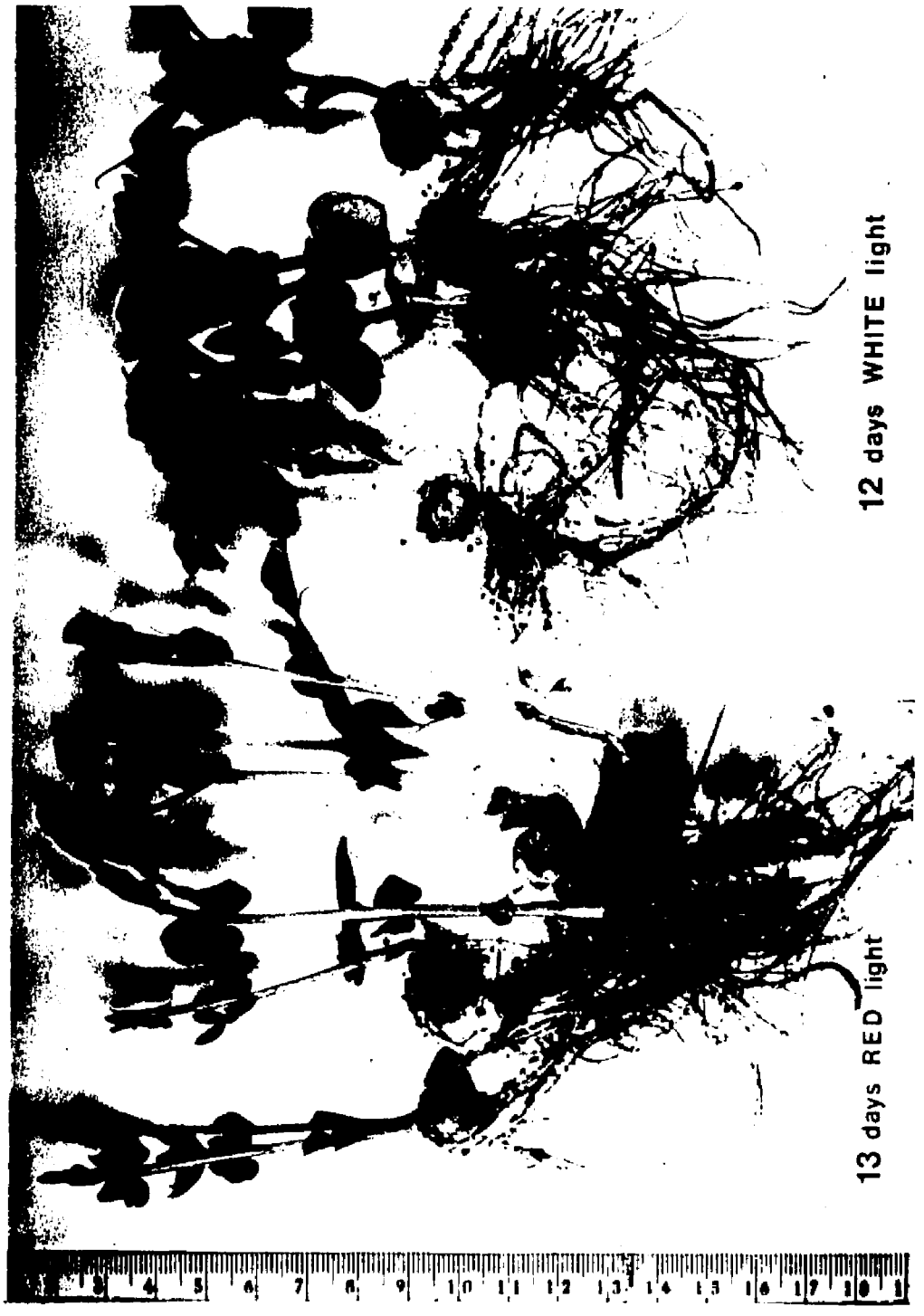


Figure 10: Comparison of seedlings grown under differing light conditions: Peas grown under white light for 13 days are compared to peas grown in red light for 12 days. The red light grown peas are more elongated and, although it cannot be seen in a black and white picture, the red light grown peas are less green.

Change in EF-G specific activity in light to dark, dark to light transferred peas:

The change in the EF-G specific activity in dark to light, and in light to dark transferred pea seedlings was investigated (Figure 11). Upon light exposure, development and differentiation start which are stalled during the growth in the dark. This feature allowed us to investigate light induction of chlEF-G in extracts of dark to light transferred pea seedlings. The EF-G specific activity of whole cell extracts of peas grown 13 days in the dark resulted in rapid induction when transferred to light. After 3 days of light exposure, the specific activity of EF-G was more than twice that of extracts of seedlings grown under continuous light. We cannot give an exact value at the zero time point, which is % EF-G specific activity for 13 day old dark-grown sample, due to lack of accurate results. The best estimate would be 7-10 % based on values obtained only with a single measurement, mentioned in the text related to Figure 8. Therefore, the increase after 24 hrs is quite significant. When peas are grown in complete darkness, they do not form true leaves until they are transferred to the light. These plants are 35-45 cm in length with greening leaflets at the very end. This uppermost part of light-induced pea seedlings was harvested, and the activity of extracts prepared from these plants was compared to the extracts of stem and leaves harvested from

Figure 11: Chloroplast EF-G activity is light-induced in 13 day old dark-grown seedlings. Peas were grown in total darkness for 13 days and then exposed to continuous white light (open triangles), or grown in the light for 13 days, and then transferred to total darkness (closed circles). Samples were harvested at the indicated times, and assayed for EF-G activity as indicated in Materials and Methods section of this chapter.

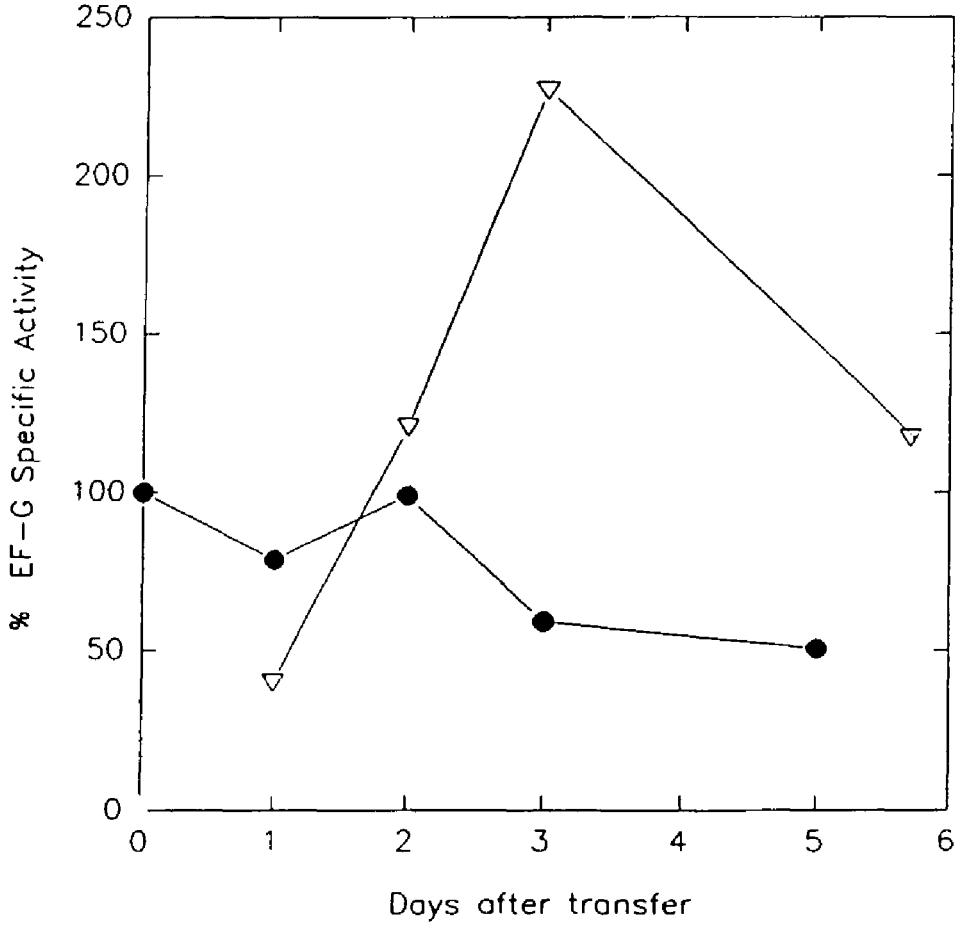

250
200
150
100
50
0
% EF-G Specific Activity
0
1
2
3
4
5
6
Days after transfer

Figure 11

light-grown peas. It should be kept in mind that the tissue compositions are different in those plants.

When pea seedlings that had been grown under continuous light for 13 days were transferred to total darkness, EF-G specific activity decreased relatively slowly (Figure 11), as compared to the induction of EF-G activity in dark-grown seedlings exposed to light. Thus, light is required for high levels of chlEF-G activity, but the absence of light does not result in rapid loss of EF-G activity. This result suggests that chloroplast EF-G is fairly stable once it is activated. Under normal growing conditions, where the seedlings experience dark cycles of 12 hrs or less, EF-G activity levels may not fluctuate very much.

Another experiment performed with 10 day old dark-grown peas harvested after 1, 2, 3 and 4 days of exposure to light showed light induction of chlEF-G specific activity (Figure 12). In this case, the chlEF-G specific activity reaches to 120 % of the maximum activity observed with the extract of 13 day old continuous light-grown pea, considered to have 100 % specific activity, after 2 days of illumination. We cannot explain the difference in results of this experiment and the one previously explained above; 13 day old dark-grown sample exhibits approximately 230 % of maximum activity when exposed to light after 3 days.

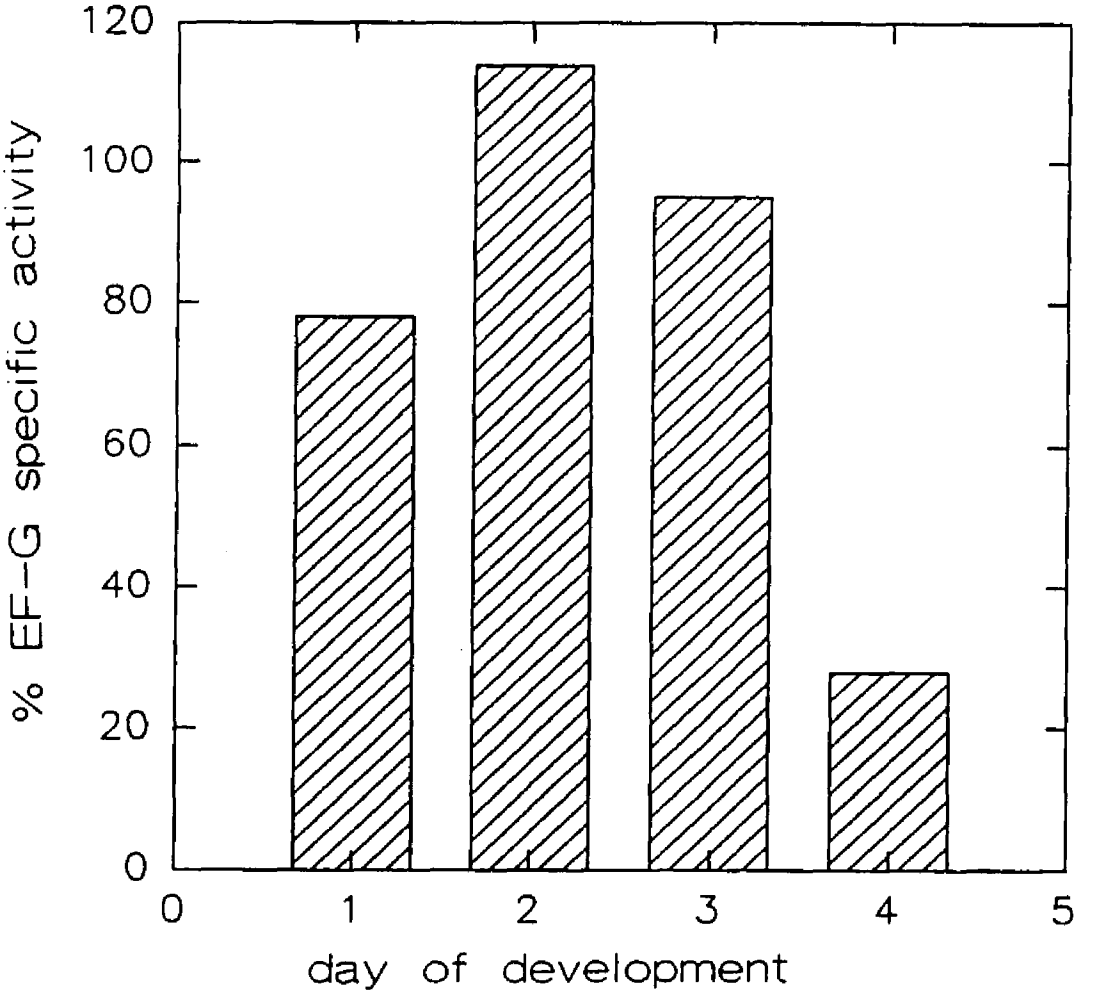
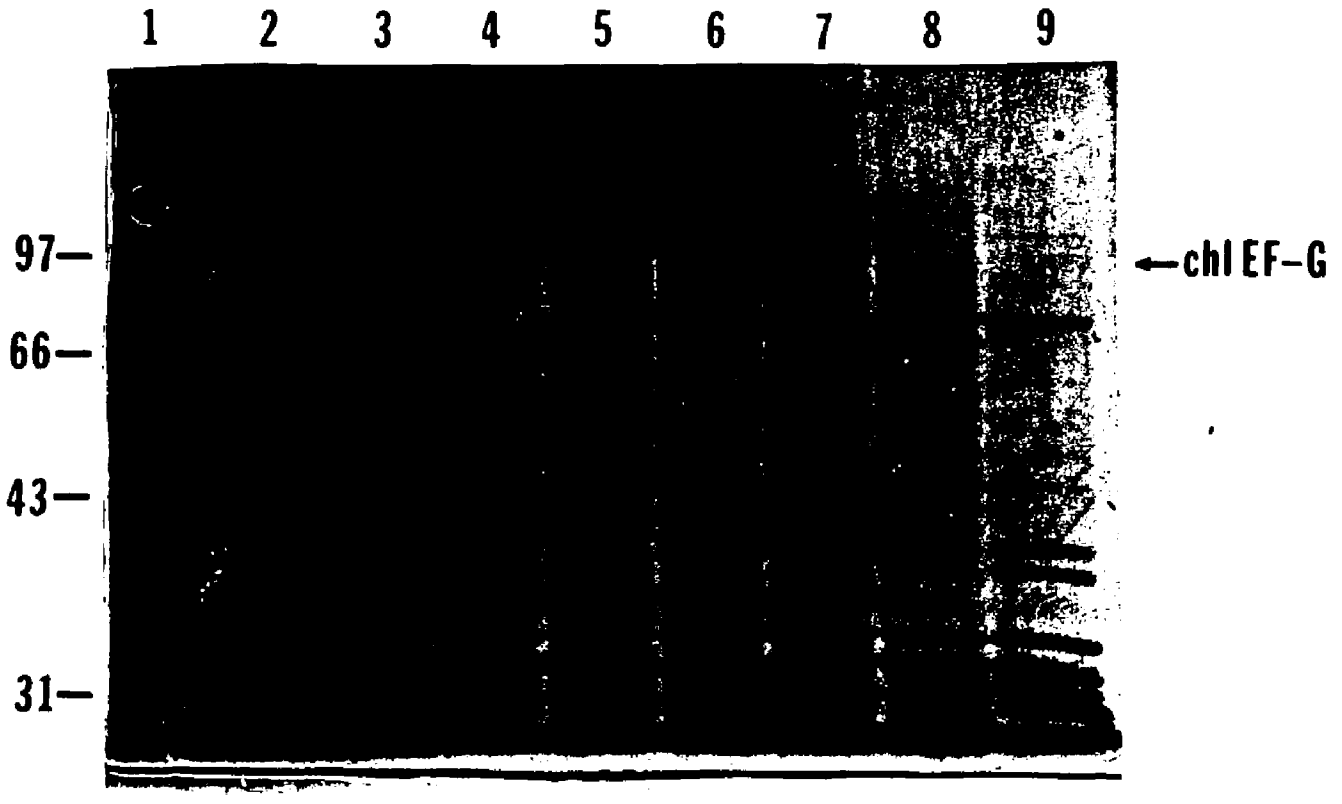


Figure 12: Chloroplast EF-G activity is light induced in 10 days old dark-grown pea seedlings. Peas grown in total darkness for 10 days and then exposed to continuous white light. Samples were harvested at the indicated time points, and assayed for EF-G activity as indicated in the Materials and Methods section of this chapter.

Change in EF-G level in the ribosomal complex:

In an attempt to demonstrate the amounts of EF-G protein at different time points in the presence and absence of light, we prepared EF-G ribosomal complexes from postribosomal supernatants of peas at different conditions. This kind of analysis could have been very difficult to do with individually purified EF-G samples due to the variability in purification efficiency. We have chosen to investigate changes in EF-G levels in the complexes formed with EF-G and ribosomes. In this way, we can eliminate the variability in preparations to a great extent. Nevertheless, it would have been very helpful to do direct and more reliable comparisons, if the photo-cross linking experiment, explained in Appendix A had worked. Figure 13 lane 5 (the upper major band corresponds to chlEF-G) clearly shows that chlEF-G level is the greatest at day 10 in the light (same time point also exhibits about maximum chlEF-G specific activity, see Figure 9). The result of this experiment is in agreement with the specific activity measurements of pea whole cell extracts. It appears that, the dark-grown samples did not exhibit the presence of significant amounts of chlEF-G, however a lower band becomes predominant at day 4 when compared to other time points; 9 and 16 days.

Figure 13: Change in EF-G levels in the ribosomal complexes of samples prepared from different time points of development of peas in the presence and the absence of light. Samples were subjected to electrophoresis on a 8 % Laemmli gel and stained with Coomassie Blue. Lane 1, molecular weight markers; 97,400; 66,200; 42,700; 31,000, lane 2, 80 μg of a crude extract of 10 days old light-grown pea seedling, lane 3, 120 μg of ribosomes, lanes 4-6, ribosomal complexes (8 μL of 100 μL suspended ribosomal complex) of 3,10, and 17 days old light-grown peas, respectively, lanes 7-9, ribosomal complexes (8 μL of 100 μL suspended ribosomal complex) of 4,9, and 16 days old dark-grown peas, respectively.


1
2
3
4
5
6
7
8
9
97—
66—
43—
31—
←chl EF-G

Rate of chlEF-G induction versus Rubisco induction by light:

To compare the rate of induction of other light-induced proteins to chloroplast EF-G, the extracts prepared in Figure 8 and Figure 11 were subjected to electrophoresis and stained so that small and large subunits of Rubisco could be visualized (Figure 14 and 15, respectively). To estimate the relative levels of both subunits, scanning densitometry of the gels shown in Figure 14A and Figure 15A was performed. The relative intensities of the subunits were obtained (Figure 14B and 15B) as explained in Materials and Methods of this chapter. The subunits of Rubisco accumulate more slowly than chlEF-G activity (Figure 14B). The amounts of Rubisco subunits are about 30-40 % of their maxima at day 8, where as the EF-G activity is close to its maximum at day 8. Similar results were obtained with the samples of the gel in Figure 15A as seen in Figure 15B. The third day of dark to light transferred sample shows the maximum EF-G activity and it decreases from then on (Figure 11). SSU and LSU continue to increase even after chlEF-G activity decreases (Figure 15B). These results indicate that the change in EF-G activity precedes the change in SSU and LSU amounts. Since active chloroplast EF-G is required for the translation of LSU which takes place in the chloroplast, it comes as no surprise that it would be light-induced more rapidly than both LSU and SSU.

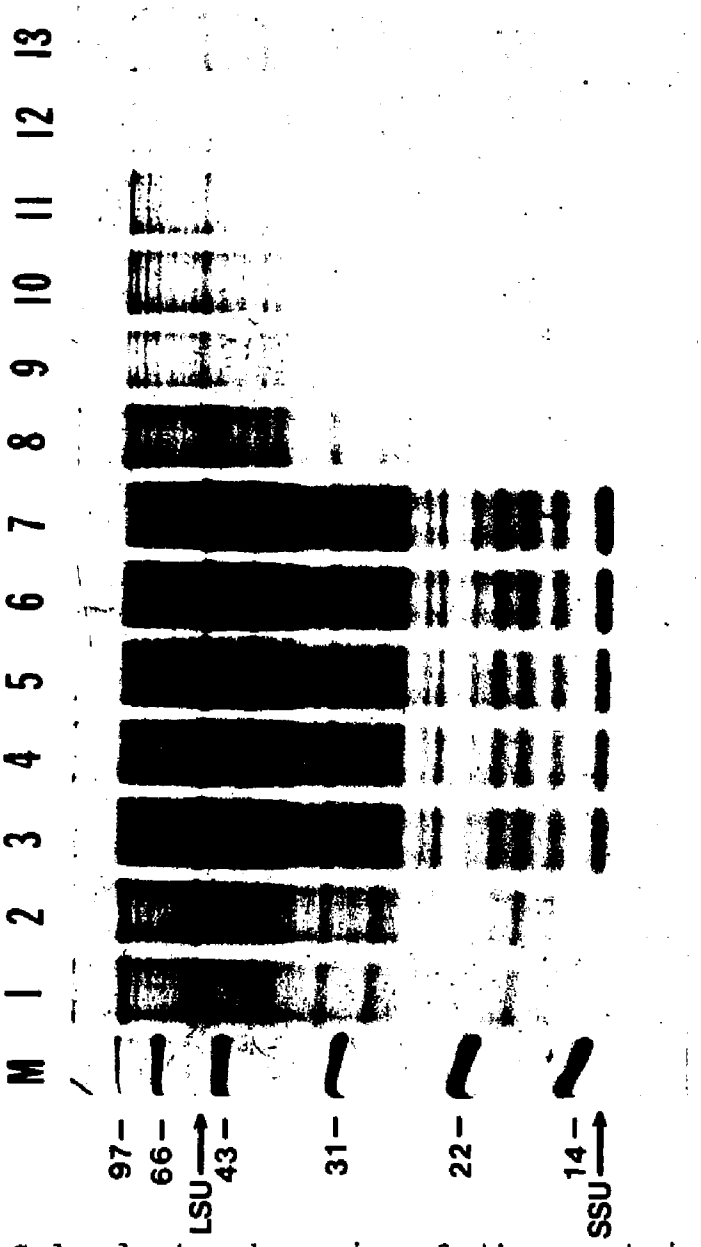


Figure 14: Gel electrophoresis of the proteins present in light- and dark-grown seedlings. **A.** Protein gel. Samples were subjected to electrophoresis on a 12% Laemmli gel and stained with Coomassie Blue. Arrows indicate protein bands which correspond to the large (LSU) and small (SSU) subunits of ribulose bisphosphate carboxylase/oxygenase. Lanes: M, markers, with molecular weights indicated on the left; 1-7, extracts from seedlings grown under continuous white light (85 μg protein) for 4, 7, 9, 11, 13 and 15 days. Lanes: 8-13, extracts from seedlings grown in the dark (95 μg).

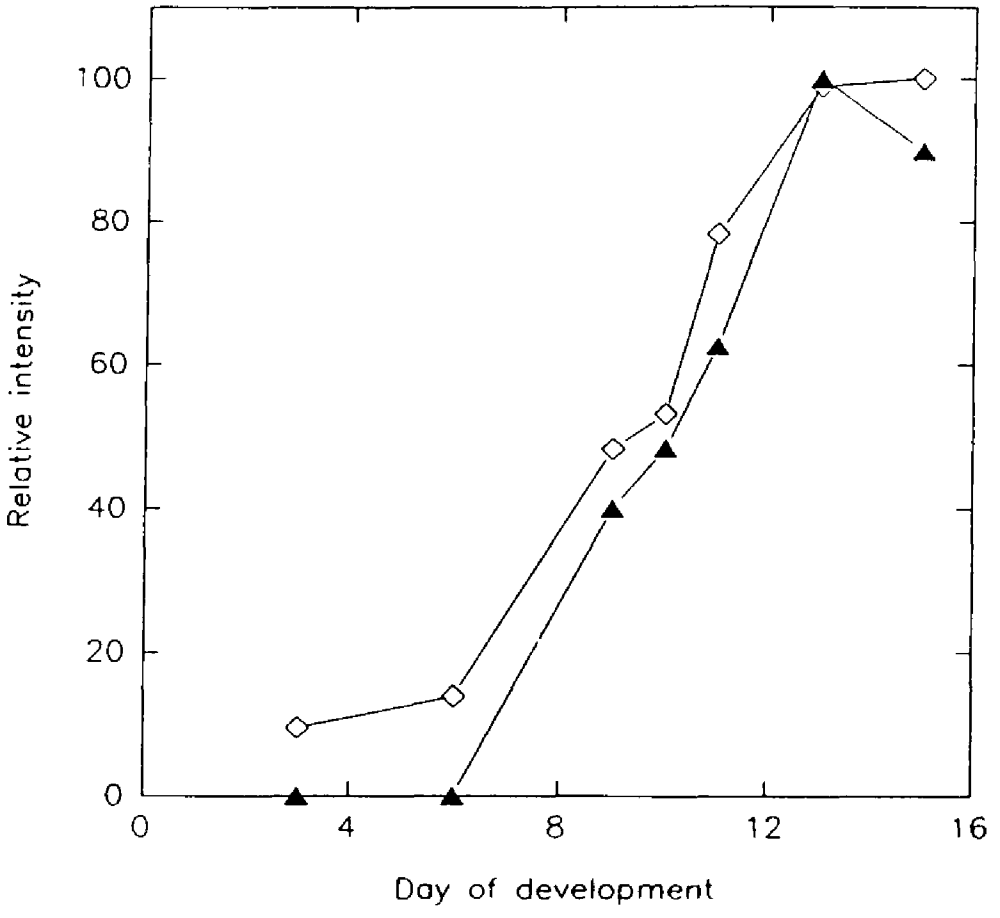


Figure 14 (continued) **B.** Scanning densitometry. Lanes 1-7 of the gel shown in A were scanned and peak heights relative to the 13-day sample (lane 6) determined. Diamonds: LSU, triangles: SSU.

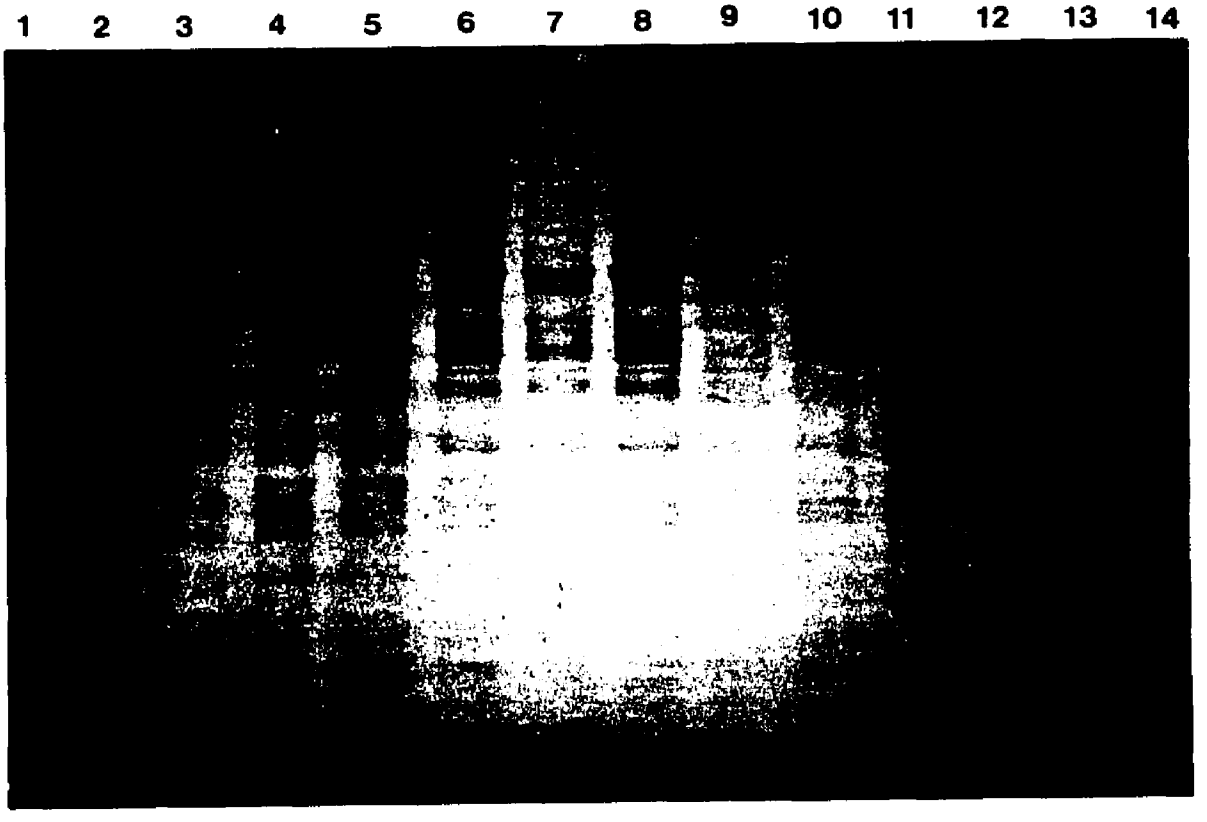


Figure 15: Gel electrophoresis of the proteins present in the dark to light, light to dark transferred seedlings. **A.** Protein gel. Samples were subjected to electrophoresis on a 12 % Laemmli gel and stained with Coomassie Blue. Arrows indicate protein bands which correspond to the large (LSU) and small (SSU) subunits of ribulose bisphosphate carboxylase/ oxygenase. Lanes: 1, 14, markers, with molecular weights indicated on the left. 2, 8: extract from 13 days light-grown (80 μg). 3, 9: extract from 13 days dark-grown (100 μg) 4-7: extracts from peas grown 13 days under light and transferred to dark for 1, 2, 3 and 5 days, respectively (80 μg). Lanes 10-13: extracts from peas grown 13 days under darkness and transferred to light for 1,2,3, and 5.7 days, respectively (80 μg).

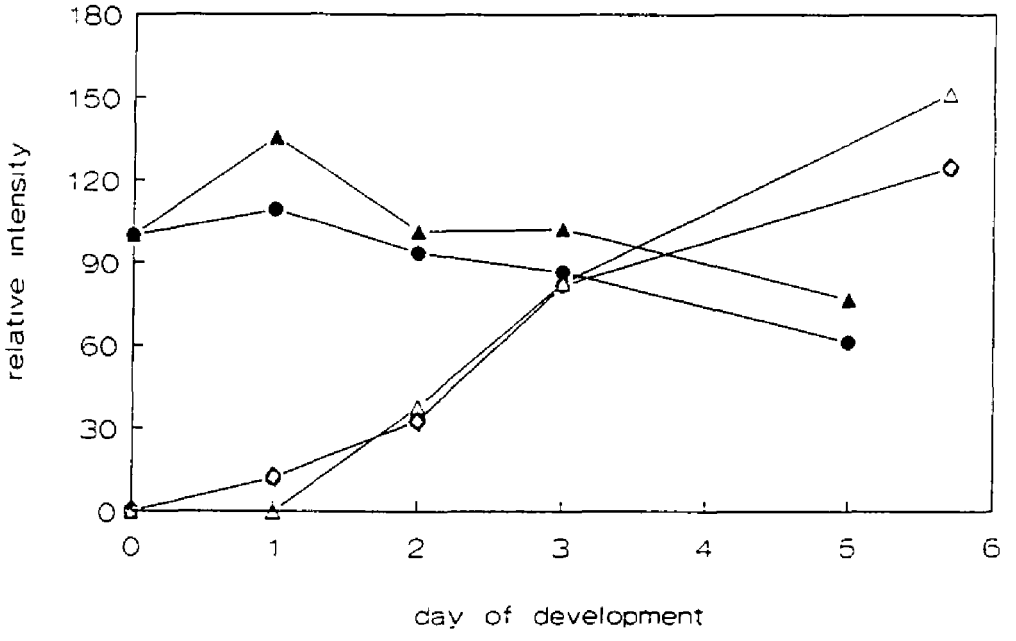


Figure 15 (continued): B. Scanning densitometry. Lanes 4-7 and 10-13 of gel shown in A were scanned and peak heights relative to the 13-day sample (lanes 2 and 8 averaged) determined. Triangles: SSU. Closed triangles, light to dark transferred seedlings, open triangles, dark to light transferred seedlings. Diamonds and circles: LSU. Closed circles light to dark, and open diamonds, dark to light transferred seedlings.

In the dark, the SSU and LSU levels decrease similarly to the decrease in chlEF-G specific activity, with a slightly slower rate (compare Figure 11, filled circles with Figure 15B filled shapes).

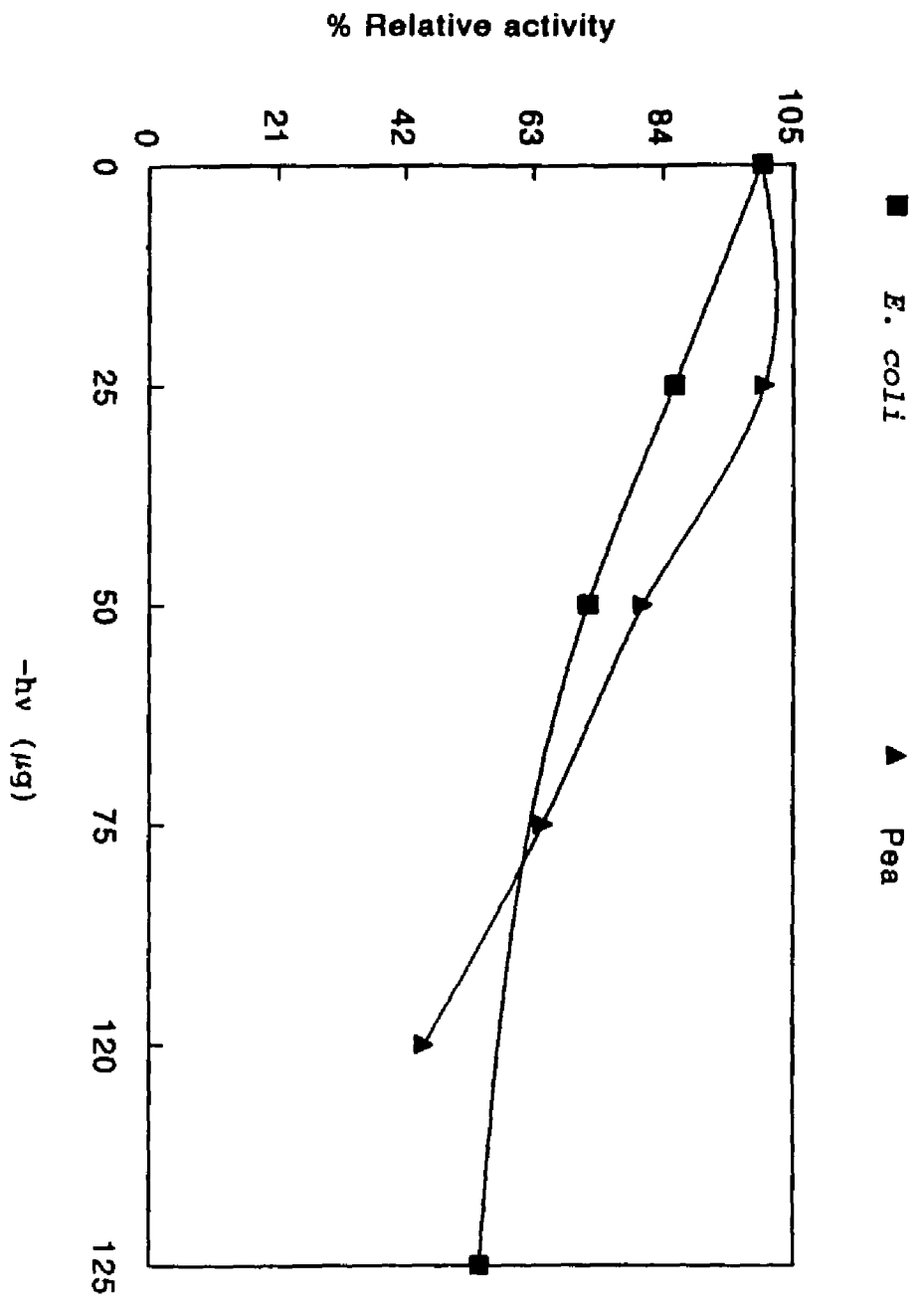
The results presented in this chapter indicate that light is required for the induction of chloroplast EF-G activity. In the absence of light, EF-G activity was low or undetectable. Red light alone was sufficient to induce the activity of this protein. As expected the induction of chlEF-G preceded that of other light induced protein(s) synthesized and/or assembled in the chloroplast.

Possibility of a specific inhibitor presence in the extracts of dark-grown peas:

The EF-G protein may be present up to a certain extent in the etioplasts, but it may be inactivated by a specific inhibitor present only in the dark-grown peas whose expression may be turned off by light. This possibility was tested by mixing experiments. To a fixed amount of fusidic acid purified chlEF-G, increasing amounts of crude extract of dark-grown pea was added. The same amounts of that crude extract were also added to *E. coli* EF-G as a control (Figure 16). The dark-grown extract inhibits the activity of chlEF-G up to 50 %, but this occurs only with very high levels of the extract. A similar inhibition was observed with the *E. coli* EF-G. These results thus suggest that the observed inhibition

is not due to a presence of a specific inhibitor, but rather may be due to a non-specific protease or RNase, interfering with the assay.

Figure 16: The effect of dark-grown extract on the purified chlEF-G. To the 4 μg of gel filtration purified chlEF-G from 10 days light grown peas and 0.21 μg of *E. coli* EF-G, 25, 50 75, 120 μg of dark-grown crude extracts were added and the activity change was measured as described in the materials and methods of Chapter II. The pure protein in the absence of crude extract was considered to be 100 % in both cases.


% Relative activity
0
21
42
63
84
105
0
25
50
75
120
125
-hv (µg)
E. coli
Pea

3.3 Discussion

The chloroplast protein synthesizing system is absolutely essential for the development of an intact, functional organelle. Its capacity is regulated in a light-dependent fashion, and for many genes encoded by chloroplast DNA, translational regulation appears to be the primary mode of gene regulation. We show that the activity of pea chloroplast EF-G is much higher in light-grown seedlings than in the dark. Light regulation of this nuclear-encoded chloroplast protein synthesis factor may play an important role in coordinating nuclear and chloroplast gene expression during chloroplast biogenesis.

It is interesting that our measurements of EF-G specific activity closely parallel measurements of pea chloroplast DNA levels by Lamppa et al., 1979. EF-G specific activity reaches a maximum around day 13 post-germination, and then decreases. The decrease observed after day 13 may be attributable to chloroplast aging, *i.e.*, chloroplast protein synthesis requirements decrease as leaves mature. Lamppa et al. noted an initial increase in the total amount of chloroplast DNA, which reached a maximum around day 7-11 post-germination. The amount of chloroplast DNA as a % of total DNA increases 2.5-fold from day 4 to day 8 (compared to an 7-fold increase from day 6 to day 8 in chlEF-G specific activity in our experiments (Figure 8)). Chloroplast DNA levels decrease as the

chloroplast mature as do EF-G levels. There is a 5.2-fold difference in % chloroplast DNA between 6 day old etiolated tissue and 8 day old light-grown sample (compared to an approximately 10-fold increase in EF-G at day 13 in our experiments, see data in the result section). Thus, increased transcription rates caused by increases in chloroplast DNA copy number occur simultaneously with an increase in chloroplast EF-G activity.

It was surprising to observe no EF-G activity or activity at barely detectable levels in the extracts from dark-grown seedlings. Not only should we have detected residual levels of EF-G activity from the etioplasts, since a low level of protein synthesis must go on even in the dark, but also our assay system should have detected mitochondrial EF-G activity. A possible explanation for that result is that TCA, which was used to precipitate polyphenylalanine synthesized by the EF-G assay, does not precipitate short chains of polyphenylalanine (we believe that the length of soluble polypeptide is in the range of 2 to probably 14 amino acid long polypeptides; dipeptides formed by EF-Tu/EF-Ts complex alone do not precipitate). There may be a threshold level of EF-G activity which generates only short chains of phenylalanine, and would therefore be undetectable in our assay. This problem makes it difficult to determine the magnitude of EF-G induction precisely. However, values obtained based on only a single point in an assay performed with dark-grown pea extracts (see

results section for data), gave us low EF-G activity. Analysis of chloroplast EF-G activity in fusidic acid affinity purified material from light-induced and dark-grown seedlings suggests a minimum of 10-fold activation by light, as presented in Chapter II.

In higher plants, red light mediated processes generally involve the phytochrome receptor. Since red light alone was sufficient to induce chlEF-G activity, this induction may also be a phytochrome-mediated response. There are two forms of the phytochrome receptor: P_r (inactive form) and P_{fr} (active form). P_r can be activated by red light to become P_{fr}. To prove phytochrome involvement, the diminished response of red light by far red light exposure should be presented. We have not performed this experiment since we did not have a far red light source to expose peas. However, it is clear that EF-G is red light induced, and it is likely that phytochrome may be involved in this induction.

Seven day old etiolated peas (*Pisum sativum* cv. Alaska) exhibit 1.9-fold induction of total RNA levels in the buds of pea seedlings upon 10 min red-light irradiation per g fresh weight (Jaffe, 1969). Thien and co-workers (Thien et al., 1975) in their studies showed that cotyledons of dark-grown mustard seedlings after a red-light irradiation increased plastid ribosomal RNA content by 1.67-fold. Therefore, an argument can be made that the induction of EF-G may be due to an overall increase in the rate of the chloroplast biogenesis,

including all components of the chloroplast transcription and translation apparatus. Light induced changes in total DNA, total RNA and total soluble protein were investigated by Sasaki and his coworkers (Sasaki et al. 1987). Pea seedlings grown in the dark for 7 days were continuously illuminated with white light for 5 days. Total DNA content per g of fresh weight decreased, however total RNA (mg RNA/mg DNA) stayed constant throughout the illumination, yet total protein (mg protein/mg DNA) increased, this means that total RNA content per total protein is decreased. We have expressed chlEF-G activities of samples as specific activities, that is, chlEF-G activity per μg of total soluble protein, and have observed at least 10-fold difference between the etiolated and light-grown peas, approximately 25-fold difference between the etiolated and light-induced peas. The increase in chlEF-G concentration in total protein is higher than the increase in levels of total protein as plants develop; indicating an enrichment of EF-G levels as plants develop in the light. Although direct comparisons cannot be made since time points, flux of light, tissue type and the source of plants are different, we can still say that the increase in EF-G specific activity (chlEF-G specific activity is expressed as units per μg of protein) may be indicative of a specific light effect on chlEF-G, since total RNA levels/mg protein decreased as opposed to an increase in EF-G activity levels/ amount of protein.

In many cases that phytochrome-mediated regulation occurs

at the level of transcription, however, this protein may also be regulated by light at the post-translational level. There may still be chlEF-G present in the dark extracts, but in an inactive form. Our mixing experiment, to study the effects of dark-grown crude extract on the activity of purified chlEF-G, suggested that there is not a specific inhibitor present, because the decrease in chlEF-G activity was no different than the decrease in *E. coli* EF-G activity (Figure 16). It still can be speculated that the protein is inactivated in the dark by some other way, for example phosphorylation/ dephosphorylation. The eukaryotic counterpart of chloroplast EF-G, cytoplasmic EF-2, in mammalian cells is activated by dephosphorylation by a specific Ca^{2+}/Calmodulin-dependent protein kinase (Ryazanov et al., 1988). Phosphorylated EF-2 is completely inactive and also inhibits the activity of non-phosphorylated EF-2. The activity measurements of phosphorylated and dephosphorylated chloroplast EF-G either by addition of a phosphatase (calf intestinal phosphatase, CIP) or crude extracts (assuming that a specific kinase or a phosphatase involved would be present and active in the crude extract) did not give any conclusive results. Future phosphorylation or dephosphorylation experiments could be performed on an enzyme immobilized column (a kinase or a phosphatase) in order to prevent possible interference of those reactions with assay components.

Chloroplast fructose diphosphatase (FDPase) activity

during photosynthesis is reported to be controlled photochemically through reduced ferredoxin (Buchanan et al., 1971). Light reduced ferredoxin activates FDPase by reducing the enzyme. This enzyme can be activated *in vitro* by addition of DTT (Rosa et al., 1984). Involvement of this kind of oxidation/reduction mechanism through ferredoxin can as well be speculated for chlEF-G. Preincubation of the EF-G-containing extract with an excess amount of DTT in an attempt to activate a component in the crude extracts from dark-grown and light-grown peas (as is the case in FDPase) did not give conclusive results (Appendix B). However, our results do not rule out the possibility of posttranslational regulation mentioned above.

CHAPTER IV

CLONING AND SEQUENCING OF A FRAGMENT OF THE GENE FOR CHLOROPLAST EF-G

4.1 Introduction

There are many examples of nuclear encoded chloroplast proteins which are transcriptionally light regulated. It is reasonable to suspect that the light induction of chlEF-G may also be at the transcriptional level.

It was necessary to obtain a specific probe to study a possible transcriptional level of light induction of chlEF-G. The probe was prepared by polymerase chain reaction (PCR). The 5'end primer of PCR was constructed from amino acid sequence of N-terminus of the protein. Because HPLC-purified material was very dilute and also low in quantity (Chapter II), it was decided to obtain N-terminal amino acid sequence from fusidic acid affinity purified material. From the cloned amplified EF-G specific cDNA/PCR product, it was possible to synthesize a RNA probe to use for northern analysis and RNase protection assays.

The outlined strategy for the analysis of the RNA probe hybridization is as follows;

The amount of mRNA should show light/dark dependence, if

the regulation is at the transcriptional level. Northern blots containing RNAs obtained from peas harvested at different conditions (dark/light vs days) will be analyzed with the RNA probe. The RNA gel will be run with the same quantities of different RNA samples in order to compare the hybridization levels. Nevertheless, the plasmid containing ß-subunit of the mitochondrial ATPase and small subunit of Rubisco will be used to normalize the quantitation of hybridization. It is critical to note that the normalization of hybridization levels is very important to claim the transcriptional level of control if there is one. Therefore comparisons should be performed on known quantities of total RNAs. Because mRNA isolation although performed with the same amounts of starting materials may vary in efficiency, it causes a difficulty in estimating the magnitudes of induction precisely. If higher levels of hybridization are observed with the RNA samples obtained from light grown or light induced pea seedlings, this then will be an indication of transcriptional (or post-transcriptional) regulation.

4.2 Materials and Methods

Sample preparation of chlEF-G for microsequencing:

The chlEF-G from 10 day old continuous light-grown pea seedlings was purified as explained in the materials and methods section of Chapter II. To concentrate chlEF-G,

fusidic acid purified (FAP) material was recomplexed with ribosomes under modified salt conditions of affinity purification procedure as detailed in the Chapter II. 2.5 mL mixture contained; 20 μM GTP, 10 mM fusidic acid sodium salt, 1,149 μg *E. coli* ribosomes, 757 μg FAP protein in Buffer A (Chapter II, Materials and Methods), the Mg^{2+} concentration increased to 20 mM with $Mg(OAc)_2$. The mixture was layered on 600 μL sucrose cushion (Buffer B; 44 % sucrose, 10 mM fusidic acid, 5.1 μM GTP, 20 mM Mg^{2+} in Buffer A). The ribosomal complex was collected by high speed centrifugation for 2 hrs at 70,000 rpm in a Beckman TLA-100.3 rotor. The pellets were washed by resuspending in 2.6 mL Buffer C (Buffer B without sucrose), overnight at 4^{o}C. The supernatant of the repeated centrifugation as above was discarded and the ribosomal complex containing chlEF-G was resuspended again by addition of 50 μL Buffer A. The complex then was dissociated by addition of 50 μL 2xTreatment Buffer of SDS gel electrophoresis (0.125 M Tris-Cl, pH 6.8, 4 % SDS, 20 % glycerol, 10 % ß-ME) and boiling for 5 min prior to loading on a 30 min prerun 7% Acrylamide SDS gel. After the completion of electrophoresis, the gel was immediately electroblotted onto the Millipore PVDF-membrane. The electroblotting conditions were as indicated in the suggested procedure supplied by the manufacturer. The membrane was stained (0.2 % Coomassie Brilliant Blue R-250 (Sigma), 45 % methanol, 10 % acetic acid) for 15 min and destained (45 % Methanol, 10 %

acetic acid) for 10-15 min. Then the band corresponding to chlEF-G was cut out for amino acid microsequencing, and stored at -80°C until sequencing.

Protein microsequencing:

The sequencing reactions were performed on the Applied Biosystems Model 470 Gas-Phase Protein/Peptide Sequencer, which uses the Edman Degradation technique, at the Biochemical Instrument Center (College of Biological Sciences, OSU) .

Oligonucleotide synthesis:

The oligonucleotides to be used as 5'-end (with Nhe I restriction site) and 3'-end (with Eco RI restriction site) primers of polymerase chain reactions and the one to be used as a hybridization probe were constructed as reasoned in the results; RevPG, G3SA, and G4L, respectively. They were synthesized on the Applied Biosystems Model 380B Synthesizer at the Biochemical Instrument Center (College of Biological Sciences, OSU), which uses α-cyanoethylphosphoramidite chemistry. The oligonucleotides were deprotected by heating at 55°C overnight and desalted by gel filtration column chromatography on a 5 mL Sephadex G-50 column.

Radioactive labeling of hybridization probes:

G4L and Hind III digested pSSU 160 (Bedbrook, J. R., 1980) containing a piece of SSU cDNA from pea were random

primer labeled by using [α^{32}P]-dCTP (3000 Ci/mmol, Amersham Corp.) and a kit purchased from Bethesda Research Laboratories (BRL). 0.5 μg of ΦX174 Hae III, DNA marker, was labeled by using T4 polynucleotide kinase (BRL) to generate the 5'-end [^{32}P] labeled molecular weight markers with [γ-^{32}P]-ATP (3000 Ci/mmol, Amersham Corp.). The unincorporated nucleotides were removed by Sephadex G-25 spin column.

Preparations of total RNA samples:

Pea seedlings were harvested at different time points as indicated in the results, fast-frozen by liquid nitrogen addition, and stored at -70°C up to a week until RNA preparations were performed, according to Chomczynski et al., 1987. Great care was taken to prevent RNase contamination. All glassware was acid washed and sterilized by autoclaving. All the solutions used were Diethyl Pyrocarbonate (DEPC) treated and sterilized as above. Gloves were worn at all times throughout the preparation.

Preparation of poly A$^+$ mRNA:

Total RNA prepared from 13 day dark-grown, and 3 day light-induced pea seedlings was used as a poly A$^+$ mRNA source, which was isolated using oligo (dT)-cellulose spin columns purchased from 5 Prime → 3 Prime Inc. according to the procedure supplied by the manufacturers.

Polymerase chain reaction (PCR):

RNA/PCR was performed as described elsewhere (Kawasaki et al., 1989), except 0.5 μL of 1 unit/μL PCR perfect match (an enhancer from Stratagene) was included in 50 μL reaction. The cDNA was synthesized from poly A^+ mRNA prepared as above by using random hexamers (Boehringer Mannheim) and Mo-MuLV reverse transcriptase (BRL). The PCR components and the enhancer were added into the same tubes where the cDNA synthesis was completed. The 30 cycles of amplification conditions were as follows; 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 1 min of polymerization at 72°C. As needed 4-6 μL of the amplified product was used as the template to generate more of the PCR specific gene fragment. 15 μL of amplified PCR products were loaded onto a 7 % nondenaturing polyacrylamide gel (Maniatis et al., 1982). The gel was stained with ethidium bromide and its picture was taken as it was visualized by a UV transilluminator.

Southern Analysis of PCR Products:

Gels containing PCR products were transferred to nylon membrane (Hybond-N, 0.45 μm, Amersham) as described in Ausubel et al., 1989. The oligonucleotide G4L was used as hybridization probe (see Results) which was random primmer labeled as explained above. DNA was covalently fixed to the membrane by heating at 80°C in vacuum oven.

Cloning of PCR product:

By hybridization of G4L (see results section of Chapter IV) probe, the 300 bp expected size PCR product was identified as chlEF-G specific. 75 μL reamplified PCR product was loaded onto 7% polyacrylamide non-denaturing gel. The 300 bp fragment was band isolated in Elution buffer (0.5 M ammonium acetate, 1 mM EDTA pH 8.0) by shaking overnight at 25°C. DNA was digested first with NheI, then EcoRI separately and cloned into pBS+ (Stratagene) cut with XbaI (there is no NheI cloning site in pBS+) and EcoRI. 20 μL ligation reaction was performed in the presence of all the band isolated insert and 0.03 μg prepared vector, 1X Ligase buffer (BRL) and 1 unit of T4 DNA ligase overnight at 16°C. Transformation of competent cells, DH5α-subcloning efficient (BRL), was conducted as suggested in the procedure supplied by the manufacturers. Mini plasmid preparations were performed according to the literature procedures (Kraft et al., 1988).

DNA Sequencing:

DNA sequencing was performed on clones which were thought to be candidates containing the insert of interest based on the molecular sizes appeared on 1 % agarose gel. DNAs were prepared by the mini plasmid preparation method referred above. DNA was sequenced by the dideoxy chain termination method of Sanger using the Sequanase version 2.0 kit (United

States Biochemical Corp.). DNA was sequenced from both ends with T7 primer and reverse primer (Promega). The isotope used was [α^{32}P]-dATP (3000 Ci/mmol, Amersham Corp.).

Preparation of RNA probe:

The sequence of the DNA insert (99 bp long) in the clone pB was oriented in a way allowing the synthesis of antisense RNA driven from the T7 promoter with T7 RNA polymerase. A large scale DNA preparation of pB from freshly transformed cells was performed with CsCl gradient centrifugation following the procedure supplied by the manufacturers of Beckman TLA 100.3 centrifuge, except the RNase treatment step was omitted in order not to contaminate later steps with RNAses. The HindIII digested pB, which is 31 nucleotides longer than the insert DNA, or AccI digested pB, which is 14 nucleotides longer than the insert DNA, were used as templates of the transcription reaction (for RNase protection assays the digested DNA was Proteinase K treated (Sigma)). The sizes of RNA probes must be 130 and 113 nucleotides, respectively. The procedure (Melton et al., 1984) was followed with some minor modifications. Digested DNAs were band isolated by using the Geneclean kit (Bio 101 Inc.). The reaction was performed as follows; 25 μL reaction mixture containing approximately 1 μg linearized DNA (as above), 0.4 mM rATP, 0.4 mM rGTP, 0.4 mM rUTP, 8 mM DTT (ribonucleotides and DTT are the components of Riboprobe Gemini System, Promega Biotech), 1X buffer, 1.5

units of RNase Block II, 10 units of T7 RNA polymerase (all three from Stratagene), and 260 μCi [α^{32}P]-CTP (800 Ci/mmol, Amersham Corp.) incubated at 37°C for 15 min and after cold rCTP addition, incubation continued for another 15 min. 5 units of RNase free DNase was added to digest the template at 37°C for 15 min. The unincorporated radioactive nucleotides were removed using 1 mL DEPC-treated and sterilized Sephadex G-25 spin column. The eluate then was phenol/chloroform extracted twice, ethanol precipitated, and 70 % ethanol washed, lyophilized and used for RNase protection assays or Northern hybridizations. $3.0x10^4$ - $5.2x10^4$ cpm (total) of RNA probes were obtained based on different preparations.

Northern analysis and hybridization:

Total RNA was prepared from peas grown under different conditions as indicated in the results section of this chapter. Total RNA samples (50 μg of RNA per lane and 3 μg of RNA ladder (BRL)) were run on 1.2 % formaldehyde agarose gel and transferred onto Hybond-N (nylon membrane, 0.45 μm, Amersham Corp.) by capillary transfer in 10X SSPE (1.8 M NaCl, 100 mM NaH_2PO_4, pH 7.4, 10 mM 10 mM EDTA , pH 7.4) overnight as in Maniatis et al., 1982, except the gel was not stained with ethidium bromide (the marker lane was cut out, stained and aligned with the other part of the gel containing samples). Northern blots were hybridized with HindIII digested pSSU160 and RNA probe prepared from AccI digested pB

as above in a hybridization solution containing 5X Denhardt's solution (0.1 % Ficoll Type 400 (Sigma), 0.1 % polyvinlpyrrolidone (Sigma), 0.1 % Bovine Serum Albumin (Sigma), 5X SSPE (0.9 M NaCl, 50 mM NaH_2PO_4, pH 7.4, 5 mM EDTA, pH 7.4), 0.5 % SDS, and 0.2 mg/mL calf thymus DNA (Sigma) at 60°C overnight. The amount of radioactivity in each lane was quantitated by radioactive scanning using Betascope 603 (Betagen Corp.).

RNase protection assay:

Approximately 10^4 cpm of RNA probe was used per 30-35 μL solution hybridizations of RNase protection assays. The hybridization solution contained (Melton et al., 1984) 100 μg total RNA, 0.4 M NaCl, 1 mM EDTA, pH 7.5, 40 mM Pipes 50 % deionized formamide (BRL), and ~10^4 cpm RNA probe. The mixture was incubated at 85°C for 10 min and transferred to 50°C for 3.5-4.5 hrs. On completion of hybridization, the temperature was lowered down to 25°C, and 300 μL RNase digestion mixture containing 0.3 M NaCl, 10 mM Tris-Cl, pH 7.4, 5 mM EDTA, pH 7.5, 80 μg/mL bovine pancreas RNase A, 2 μg/mL RNase T1 (Sigma Chemical Comp.) was added and incubated at 37°C for 1 hr. 20 μL of 10 % SDS and 10 μL of 10 mg/mL proteinase K (Sigma) were added, the mixture was incubated for 30 min at 37°C. Hybrids were then phenol/chloroform extracted twice, ethanol precipitated, 70 % ethanol washed, and lyophilized. Prior to loading onto the 7 % polyacrylamide

denaturing gel (containing 8 M urea) electrophoresis, hybrids were denatured in 20 μL solution containing 97 % deionized-formamide, 0.1 % SDS, 10 mM Tris-Cl, pH 7.0 by heating at 85°C for 2 min. The extent of protection was analyzed on Betascope 603 (Betagen Corp.), by measuring ß radiation.

4.3 Results

N-terminal amino acid sequence:

To explain the rationale for preparation of a sample for amino acid microsequencing, we have demonstrated that *E. coli* ribosomal proteins do not comigrate with chlEF-G on an SDS-Gel. We have compared the separation of proteins of *E. coli* ribosomes (prepared as in Materials and Methods, Chapter II) and EF-G, in this case *E. gracilis* chlEF-G (prepared from light-induced *E. gracilis*, as in Breitenberger et al., 1979a). Figure 17 presents the comparisons of these proteins. *E. gracilis* chlEF-G, lane 4, the major band, migrates at ~85,000. Lanes 2,3,5 and 6 are *E. coli* ribosomal proteins, revealing the absence of any protein migrating at 85,000 molecular weight range.

The fusidic acid affinity purified material, although only partially purified, was pure enough to yield the sequence of 15 amino acids from the N-terminus, when the protein band corresponding to the chlEF-G (M_r ~86,000) was cut out after transfer to the membrane for microsequencing. At the

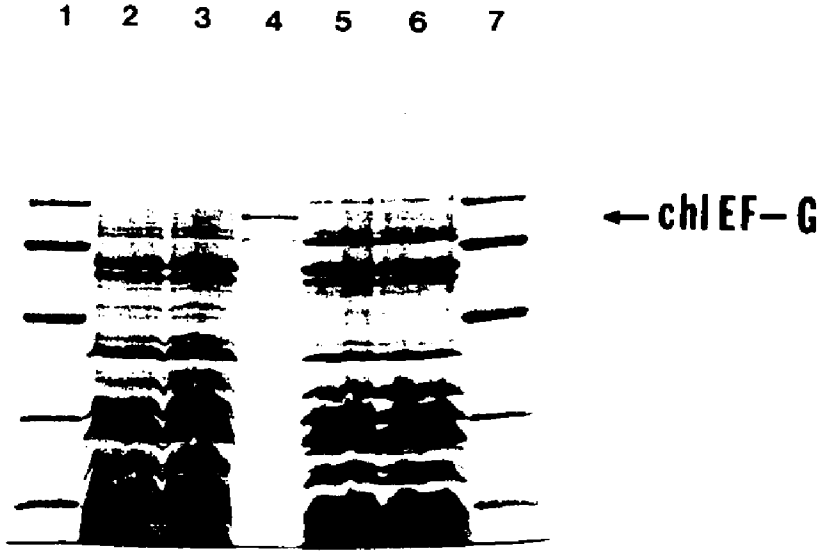


Figure 17: Comparison of molecular weights of *E. coli* ribosomal proteins and *E. gracilis* chlEF-G. Protein gel, samples were subjected to electrophoresis on a 10 % Laemmli gel and stained with Coomassie Blue. Lanes 1,7 are molecular weight markers; top to bottom, 97,400 ;66,200; 42,700; 31,000; 21,500. Lanes 2, 3 and 5, 6 are different ribosome preparations; lane 2 (~190 μg) ribosomes (prep1) and lane5 (~130 μg) ribosomes (prep2). Lanes 3 (same as lane 2), 6 (same as lane 5) ribosomes and added fusidic acid (7 mM/lane fusidic acid was added prior to loading to distinguish the difference, if any, in the electrophoresis of ribosomal proteins in the presence of fusidic acid, because there is fusidic acid present in the sample prepared for amino acid microsequencing).

molecular weight of chlEF-G, which is 86,000, there was no other protein to possibly contaminate it; all the ribosomal proteins of *E. coli* are smaller than 86,000, *i.e.* the largest molecular weight protein of *E. coli* is S1, 61,159 (Wittmann-Liebold, 1986, also see Figure 17). In Chapter II Figure 4A lane 2 shows the control for the fusidic acid affinity purification step; in the absence of crude extract, the ribosomal pellet releases two proteins of different molecular weights than that of chlEF-G.

The sequence obtained is as follows;

Ala-Thr-Glu-Asp-Gly-Lys-Arg-Ala-Val-Pro-Leu-Lys-Asp-Tyr-Arg

This sequence was obtained from 15 pmol of membrane transferred protein. The identification of the fifteenth amino acid was not as certain as the first fourteen because of high signal to noise ratio. Later this arginine was confirmed independently by DNA sequencing. The chlEF-G transit peptide cleavage site is alanine, in agreement with Gavel et al. 1990, who noted that alanine is the conserved cleavage site motif in chloroplast transit peptides.

Oligonucleotide construction:

Part of this sequence information was used to construct the 5'end primer of PCR. Only seven amino acids from the N-terminus were included with addition of an NheI site at the 5'end for the oligonucleotide. The oligonucleotide, RevPG, a 30-mer, was constructed by taking into consideration the codon

usage in pea (Wada et al., 1990), in which the AGR arginine codons are the most frequently used.

```
                    Nhe I
RevPG:    5'  CTA GCTA GCN ACN GAR GAY GGN AAR AGR GC  3'
                      Ala Thr Glu Asp Gly Lys Arg Ala
                  (N-terminus)                  (C-terminus)
```

The oligonucleotide has 1,024-fold degeneracy with no more than 3 nucleotide homology that might allow primer-dimer formation at the 3' end. Only 23 nucleotides of 30 hybridize to the cDNA template.

The 3' primer of PCR was constructed from a perfectly conserved sequence of EF-Gs (Figure 21) as G3SA with an EcoRI site. Again, since the GGA/T codons for glycine are the most abundant in pea (Wada et al., 1990), these were the codons used at the 3' end of the primer. G3SA is a 22-mer.

```
                  EcoR I
G3SA:     5' GCGAATTC AA RTC NAC RTG WCC  3'
                      Phe Asp Val His Gly
               (C-terminus)        (N-terminus)
```

G4L was constructed from another very highly conserved sequence of EF-Gs to use as a hybridization probe for specifying PCR product.

```
G4L,
template seq.: 5 'CTCG AGT NGT YTT NCC NGC RTC DAT RTG NGC 3'
                       Thr Thr Lys Gly Ala Asp Ile His Ala

                     (C-terminus)                (N-terminus)
```

PCR strategy:

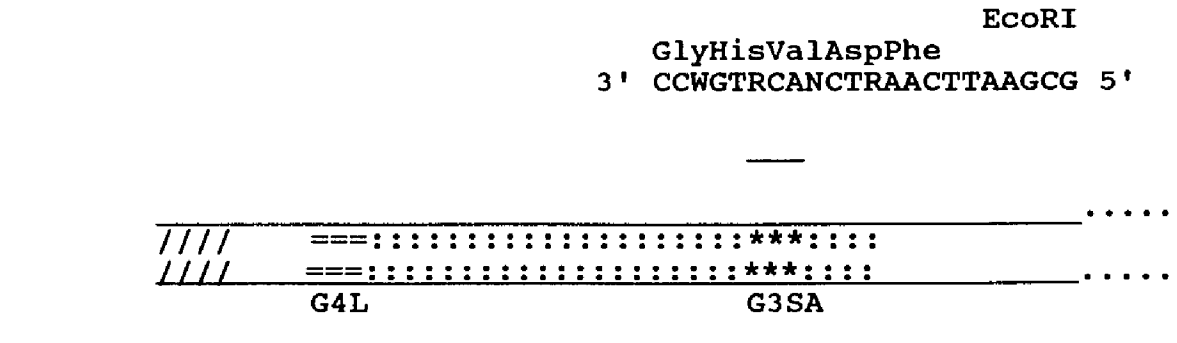
Figure 18 summarizes the overall PCR strategy.

The expected size of the PCR product should be approximately 300 bp. G4L hybridization of PCR products showed that an EF-G specific 300 bp product was amplified (Figure 19 shows the reamplified material).

The NheI and EcoRI digested PCR product was cloned into XbaI and EcoRI digested pBS+ as described in Materials and Methods. Because there was an EcoRI restriction enzyme site within the chlEF-G DNA PCR fragment only 99 bp of 300 bp could be cloned (its nucleotide sequence is given in Figure 20). The amino acid sequence derived from DNA sequencing of pea chlEF-G is shown in Figure 21. The remainder of the 15 known N-terminal amino acids following the 8 amino acids used for the 5' end primer perfectly match to the sequence obtained by direct sequencing. The later part shows a very high homology to *E. coli* EF-G and the others. The homology somewhat decreases when compared to cytoplasmic EF-2s as would be expected.

Northern analysis:

RNA probe prepared from the vector containing PCR fragment was used in Northern analysis (Materials and Methods). Although much time and effort were spent, northerns containing total RNAs of 1 day and 10 day old light-grown



5' CTAGCTAGCNACNGARGAYGGNAARAGRGC 3'
AlaThrGluAspGlyLysArgAla
NheI

/// Sequence derived by direct sequence of purified chlEF-G

=== *** Highly conserved sequences

::: GTP-binding domain

Figure 18: PCR Strategy

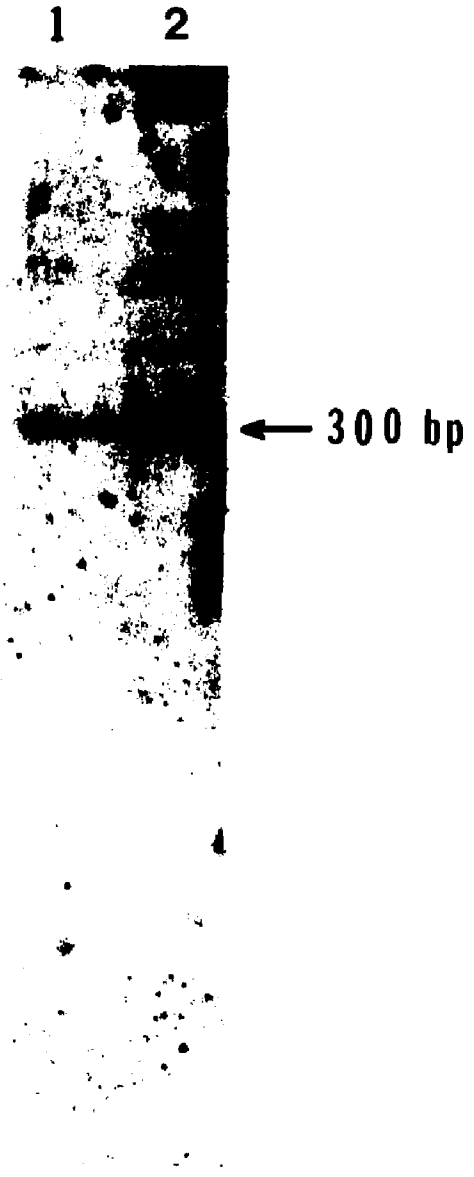


Figure 19: Autoradiogram of G4L hybridized Southern transfer of PCR products. Lane 1; Reamplification of PCR product obtained using a cDNA template synthesized from poly A^+ selected RNA of 13 days dark-grown followed by 3 days light-induced pea seedlings. Lane 2; Reamplification of band isolated PCR product of template used for Lane 1 (300 bp PCR product obtained from the template of reaction in lane 1 was band isolated and used as a template for reamplification).

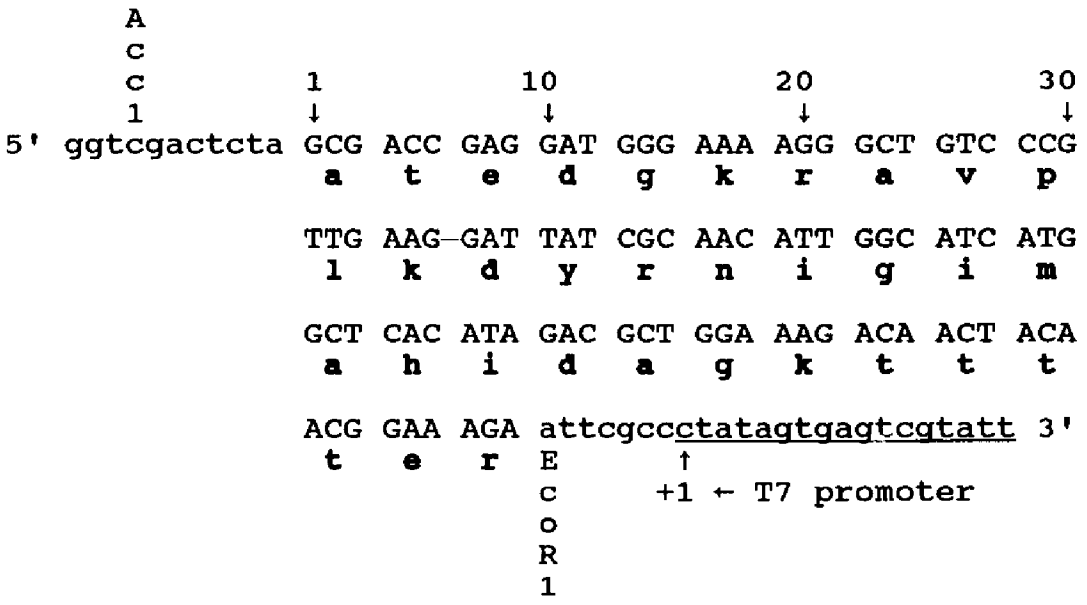


Figure 20: Partial cDNA sequence of pea chlEF-G. The DNA sequence is given as triplet codons in capital letters, lower case letters are plasmid sequence (pBS+), bold lower case letters are amino sequences. AccI and EcoRI restriction sites are indicated on the sequence. The underlined sequence is the complementary sequence of T7 primer.

Figure 21: N-terminal amino acid sequences derived by DNA sequencing for translocases from *E. coli*, *M. luteus*, *T. thermophilus*, *M. vannielii*, and hamster (Zengel et al., 1984, Ohama et al., 1987, Yakhnin et al., 1989, Lechner et al.,1988, Kohno et al., 1986) are aligned below. The N-terminal amino acid sequence of pea chloroplast EF-G is given. The underlined amino acids in the *E. coli* sequence have been implicated in guanine nucleotide binding. Dashes indicate that the sequence is the same as that of *E. coli*, and spaces indicate deletions in one sequence relative to the others.

```
1.  Escherichia coli EF-G                          MARTTPIARYRNIGISAHIDAGKTTTTE
2.  Micrococcus luteus EF-G                          ML-DLHKV-----M------------
3.  Thermus thermophilus EF-G                      MAVKVEYDLK-L-----A------------
4.  Methanococcus vannielii EF-2  MGRRAKMVEKVKSLMETHDQI--M--C----H----LSD
5.  Hamster EF-2                     MVNFTVDQIR-IMDKK-NI--MSVI--V-H--S-L-D
6.  Pea chloroplast EF-G                 ATEDGK-AV-LKD------M------------
                                                                phosphate binding

1.  RILFYTGVNHKIGEVHDGAATMDWMEQEQERGITITSAATTAFWSG          MAKQYEPHRINIID
2.  -H---------L--T---G--T------K----------V-C--ND                  -Q-----
3.  ---Y---RI-------E------F----R-------A-V--C--KD                  -------
4.  NL-AGA-  MISKDLAGDQLAL-FD-E-AA-----YA-NVSMVHEY              NGKEYL--L--
5.  SLVCKA-  IIASARAGETRFT-TRKD----C---K-T-ISL-YELSENDLNFIKQS-DGSGFL--L--
6.  -

1.  TPGHVDFTIEVERSMRVLDGAVMVYCAVGGVQPQSETVWRQANKYKVPRIAFVNKMDRMGANFLKVVNQ
2.  N-------V-----L-------A-FDGKE--E----------D--D----C------KL--D-YFT-DT
3.  ---------------------IV-FDSSQS-E----------E---------A----KT--DLWL-IRT
4.  -------GGD-T-A--AI----V-C---E--M--T---L---L-E--KPVL-I--V--LINELKLTPEE
5.  S------SS--TAAL--T---LV-VDC-S--CV-T---L---IAERIKPVLMM-----ALLELQLEPEE
    phosphate binding
```

Figure 21

peas, 1 day light induced peas after 13 days of dark-growth, and 10 day old dark-grown peas resulted in no specific hybridization with this specific RNA probe. RNase protection assays also did not give conclusive results. It was decided that the chlEF-G signal was very low, and I attempted to perform the hybridization on poly A^+ selected RNAs of two samples (Materials and Methods); 1 day light induced after 13 days of dark-growth, and 10 day old dark-grown peas. The blot of a RNA gel containing 1/4 of the poly A^+ RNA prepared from 1.5 mg total RNA starting materials was prehybridized and hybridized at 60°C overnight, four day exposure to the film did not show any hybridization. We have, as a control, a specific probe for Rubisco SSU, which is transcriptionally light-induced. The intactness of the total RNA samples used were tested by SSU hybridization. The signals that were originally expected to be obtained from the total RNA samples were then planned to be normalized with SSU signals.

4.3 Discussion

We were successful in obtaining the amino acid sequence for 15 amino acids from the N-terminus of chlEF-G. This information was used in the construction of a PCR primer which resulted in amplification of a chlEF-G cDNA fragment. The DNA sequence yielded an amino acid sequence of 33 amino acids and confirmed the sequence obtained from direct sequencing.

Furthermore, the 86,000 molecular weight band seen on SDS gel was correctly attributed to chlEF-G. From this short sequence we have obtained valuable information for the extent of homology among EF-Gs, *i.e.* 21 amino acids out of 33 perfectly match to *E. coli* EF-G.

Northern hybridization experiments did not give the expected result (mentioned in the introduction section of this chapter). There may be many reasons for why it did not work; during the isolation, RNA may have been degraded, the efficiency of blotting may be low, the probe size may be insufficient, signal may be very weak. Northern hybridizations were performed with DNA probes as well as an RNA probe. For example; 5' end labeled, random primer labeled, poly A^* tailed 300 bp long band isolated PCR product were all used as probes. Before we obtained PCR fragment, the oligonucleotide, PEAG-1, synthesized from N-terminal amino acid sequence was also used as a hybridization probe. Nevertheless, we could not detect the transcription signal of chlEF-G. Quantitative PCR analysis of cDNA template obtained from peas grown under different conditions may be an alternative way of studying RNA level differences.

CHAPTER V

CONCLUSION

We have shown that;

i. the extracts of dark-grown peas have very low basal levels of EF-G specific activity,

ii. red light alone is sufficient to induce EF-G to its white-light induced maximum. Although this observation does not prove that the induction is phytochrome mediated, it strongly suggests that it may be the case,

iii. extracts of dark to light transferred peas show more than two-fold further induction,

iv. partially purified chlEF-G from dark-grown and light-induced peas resulted in 14-fold activity increase which is two fold higher than the phytochrome-mediated induction of chloroplast ribosomal RNAs. Therefore, light has rather a specific effect on the induction of this protein rather than a mere developmental effect.

Although we think that the results are sufficient to say that EF-G is light regulated, one more important experiment should be done to prove that chloroplast EF-G levels are limiting the rate of elongation in the etioplasts. It should

be shown that the addition of purified chloroplast EF-G increases the protein synthesis levels in extracts from isolated etioplasts *in vitro* in the presence of ATP. We know from mixing experiments (Chapter III) that there is probably no specific inhibitor of EF-G in dark-grown pea extracts, therefore the increase in the capacity of protein synthesis in etioplast would be the indication of low quantity of EF-G presence due to either transcriptional or posttranscriptional limitations in the etioplasts, or protein may be present in the inactive form and activated posttranslationally by light. The eukaryotic counter-part of elongation factor G (EF-2) in mammalian cells is activated by dephosphorylation (Ryazanov et al., 1988). In any case, the light induction may still be phytochrome mediated, which also should be shown by reversed red light response upon exposure to far red light.

An explanation of why chloroplast protein synthesis is regulated by light at the elongation cycle rather than initiation and, by EF-G rather than EF-Tu/Ts can be as follows: If the regulation were to be at the initiation level a regulation of an initiation factor would result in nonuniform reduction in protein synthesis due to variable efficiency of initiation factors for different mRNAs. This would be very detrimental to the cell, some of the proteins cannot even be synthesized, *i.e.* if the affinity of the initiation factor that were regulated is very low for a specific mRNA. However having the regulation at the

elongation step blocks the protein synthesis more generally in the absence of light. If it were to be EF-Tu/Ts complex instead of EF-G, its reduction, since the relative ratio of EF-Tu to aminoacylated tRNAs would be low, could lead to increased misincorporation of amino acids, again more detrimental effect. Although low, still protein synthesis is taking place in the etioplast, therefore it is logical to have the regulation at the elongation level. Results of this project are very likely to initiate new areas of studies in our laboratory.

i. by using this EF-G specific probe the gene may be isolated or it may be possible to make a longer size probe from this one by inverse PCR techniques which are claimed to result in amplification of fragments up to 10 kb in size.

ii. it would be very interesting to investigate the possible involvements of cis- and trans-acting elements in the regulation of gene expression by studying the promoter region of this gene and comparing it to the other genes whose regulation is by light and phytochrome mediated.

iii. by *in vitro* expression of this gene or expressing it in transgenic plants by fusing to a reporter gene, it may be possible to study the light induction, organ specific expression, and organellar transport mechanism further.

I think a complete study of these aspects will lead to very valuable answers. I would like to think that my project will lay the foundation for this future research.

"the time is ripe for the search for chloroplast proteins that regulate the activity of chloroplast genes and/or the nuclear genes on which chloroplast 'life' depends."

O. Ciferri, 1978

APPENDIX A

Additional data for Chapter II

Attempts to identify chlEF-G in the crude extracts:

Prior to the HPLC-purification of pea chlEF-G, two approaches were under taken to identify chlEF-G.

Labeling of chlEF-G with [α^{32}P]-GTP: After the electrophoresis and following electroblotting to PVDF membrane of crude light- and dark-grown pea extracts (75 μg/lane), the membranes were incubated in a 10 mL buffer (50 mM Tris-HCl, pH 7.5, 0.3 % Tween 20, 2 μM $MgCl_2$, 1 mM DTT, 1.0 μCi α^{32}P-GTP/mL) in the absence of fusidic acid or in the presence of fusidic acid (4 mM) or in the presence of fusidic acid (4 mM) and *E. coli* ribosomes (36 μg/mL), for 30 min at 20°C. After washing in the same buffer (no radiolabelled GTP, no fusidic acid, no ribosomes), the dried membranes then were exposed to X-ray film for 12 hrs and 10 days. This general procedure was used for the detection of lower molecular weight GTP-binding proteins (Bhullar et al., 1987). Pea chlEF-G and mitoEF-G were not labeled specifically at these three different conditions. Autoradiograms did not display a band where chlEF-G was expected to be, although three was at least

one smaller labelled band (data not shown). The experiment was repeated, again did not give a positive result. The very same labeling method was tried with the same samples as above and including fusidic acid affinity purified EF-Gs of light- and dark-grown peas. This time samples were electrophoresed on non-denaturing gels (so that EF-G would have a native form). The result was not positive.

Photo-cross-linking of [α^{32}P]-GTP to chlEF-G: UV-light catalyzes the formation of covalent cross-links between nucleic acid and proteins (Smith, 1962). The first step in the mechanism of GTP hydrolysis by EF-G is the formation of a binary complex. The effects of uv irradiation were studied on this binary complex (Rohrbach, et al., 1977). We have adapted this photo-cross linking technique in attempts to detect EF-G in crude extracts. The crude extracts (35 μg) of light- and dark-grown peas as well as partially affinity purified EF-Gs (20 μg) from those extracts were photo-cross linked by exposing the mixture containing protein, 20 μCi [α^{32}P]-GTP/sample, 5 mM $MgCl_2$ to 254 nm uv hand lamp (flux was not determined) for 5 min and 45 min at 1 cm distance. The samples then were electrophoresed (8% acrylamide SDS-gel). The autoradiograph (not shown) of the gel did not show photo-cross linked EF-Gs. The uv light fluence might not have been efficient, since I could not obtained photo-cross-linked EF-Gs.

APPENDIX B

Additional data for Chapter III

Attempts in investigation of a possible posttranslational regulation of chlEF-G

DTT effect on EF-G: As mentioned in the discussion part of Chapter III, light reduced ferredoxin activates chloroplast fructose diphoshatase (FDPase) activity by reducing the enzyme. This enzyme can be activated *in vitro* by addition of DTT (Rosa et al., 1984). Therefore, we have tested the effect of high concentrations of DTT on EF-G with an expectation that preincubation of assay mixture with an excess amount of DTT would activate a component, and in turn increase EF-G activity in the dark-grown pea extracts. This expectation was based on the assumption that EF-G is present in an inactive form in the dark-grown peas and that there may be a component which is stimulated by DTT reduction. Table 9 presents somewhat nonconclusive results. DTT preincubation did not increase the activity as expected. Because the component in question might have been lost during the ammonium sulfate fractionation, this result is not a conclusive one.

Table 9: DTT effect on EF-G activity.

	incubation	DTT (μM)	cpm ([^{14}C]-Phe)
Sample A (1.10 μg)	no	no	9383
" (0.11 μg)	no	no	1853
" "	no	50	1440
" "	yes	50	2230
Sample B (15.0 μg)	no	no	81.9
" "	no	50	98.8
" "	yes	50	173

Sample A; *E. coli* EF-G (control), Sample B; 13 days dark-grown pea ammonium sulfate fractionated postribosomal supernatant. The samples were incubated or not, in the absence or in the presence of DTT (50 μM) then assayed for EF-G activity.

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