論文 / 著書情報
Article / Book Information

題目(和文)	
Title(English)	Studies on the Mechanism of Regulation of 5-Aminolevulinic Acid Synthesis in Cucumber (Cucumis sativus)
著者(和文)	増田建
Author(English)	TATSURU MASUDA
出典(和文)	学位:博士(理学), 学位授与機関:東京工業大学, 報告番号:乙第2715号, 授与年月日:1995年1月31日, 学位の種別:論文博士, 審査員:
Citation(English)	Degree:Doctor of Science, Conferring organization: Tokyo Institute of Technology, Report number:乙第2715号, Conferred date:1995/1/31, Degree Type:Thesis doctor, Examiner:
学位種別(和文)	博士論文
Type(English)	Doctoral Thesis

Studies on the Mechanism of Regulation of 5-Aminolevulinic Acid Synthesis in Cucumber (*Cucumis sativus*)

A DISSERTATION

submitted in partial fulfillment of

the requirement for the degree

DOCTOR OF SCIENCE

TATSURU MASUDA

Department of Biological Sciences

Faculty of Bioscience and Biotechnology

Tokyo Institute of Technology

1994

CONTENTS

ABBREVIATIONS

AFM	acifluorfen methyl
ALA	5-aminolevulinic acid
BA	N^6-benzyladenine
bp	base pair(s)
Chl	chlorophyll
DTT	1,4-dithiothreitol
DPE(s)	diphenyl ether compound(s)
Glu	glutamate
GSA	glutamate 1-semialdehyde
kDa	kilodalton
LA	levulinic acid
NF	nitrofluorfen
Proto	protoporphyrin IX
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSC	0.15 M sodium chloride + 15 mM sodium citrate
TCA	trichloroacetic acid

Chapter I

Introduction

Chlorophyll (Chl) is one of a group of tetrapyrrole molecules which play an essential role in photosynthesis of plant and phototrophic bacteria. Other members of the group include heme, siroheme (the prosthetic group of nitrite and sulphite reductases) and the bilin chromophores, and these molecules are found in most compartments within the cell: chloroplasts, mitochondria, associated with the endoplasmic reticulum, and in the cytosol. All the compounds have the common tetrapyrrole structure, differing from one another by the nature of the ring substituents, the metal ion which is coordinated into the circular molecules, and the presence of adducts such as isoprenoid chains. It is likely that all of these molecules are associated with proteins *in vivo*, and this alters their functions, as shown by the facts that the same tetrapyrrole molecule can have quite different properties depending on the protein with which it is associated, for instance, the redox potentials of protoheme in the various cytochrome *bs*, or the absorption maxima of Chl *a* in different antenna complexes.

Tetrapyrrole pigments are derived from small precursor molecules that are assembled into tetrapyrroles by several enzymes acting in sequence (Fig. I-1). The first committed precursor that is common to the biosynthesis of tetrapyrroles in all species is the five-carbon molecule, 5-aminolevulinic acid (ALA). All of the carbon and nitrogen atoms of the tetrapyrrole macrocycle are derived from eight molecules of ALA (Castelfranco and Beale, 1983; Beale and Weinstein, 1990). The earliest steps of tetrapyrrole biosynthesis result in the formation of ALA from general, noncommitted metabolic intermediates. This fundamental role of ALA as the first committed precursor in porphyrin

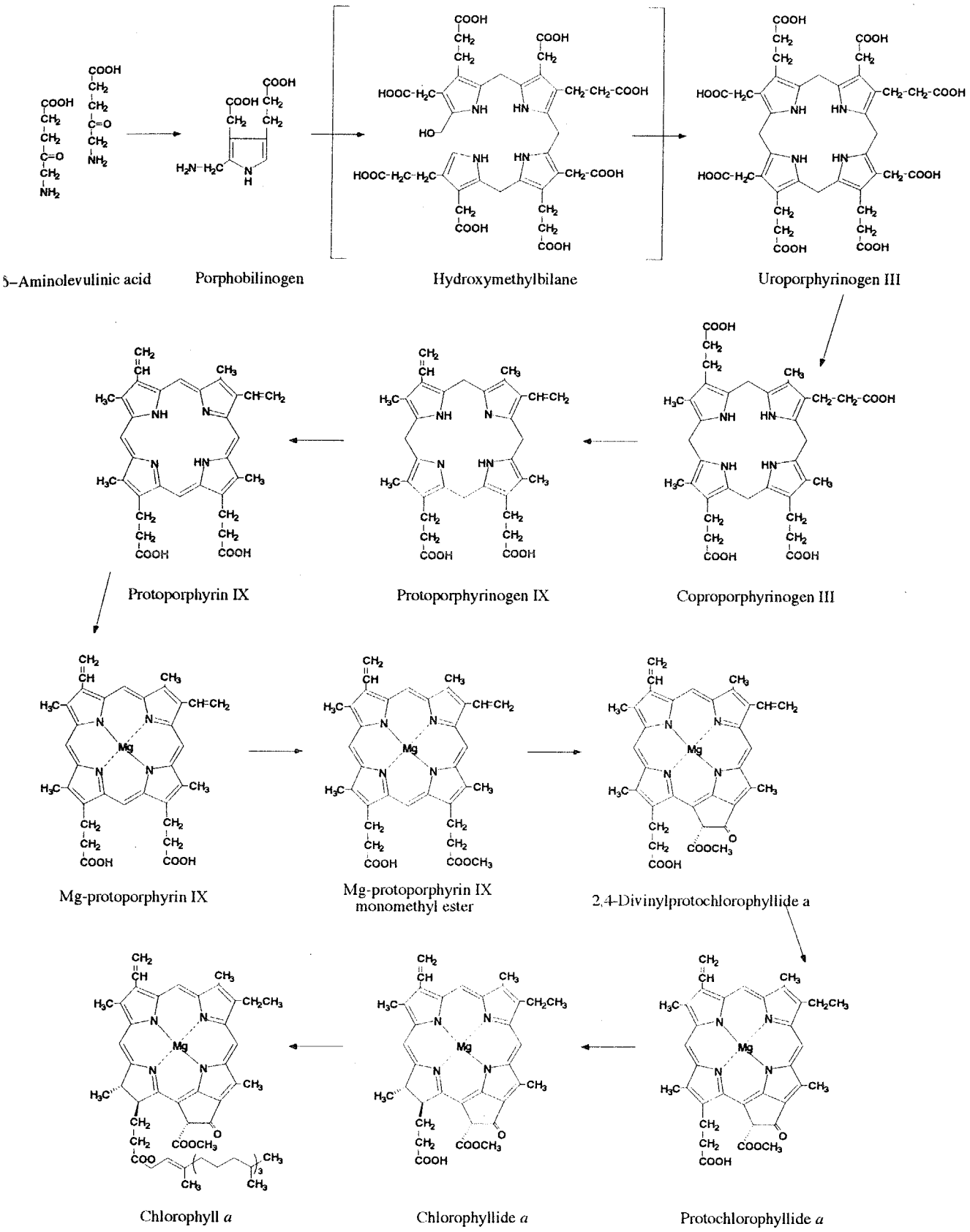


Figure I-1. The pathway from ALA to Chl *a* with the principal intermediates.

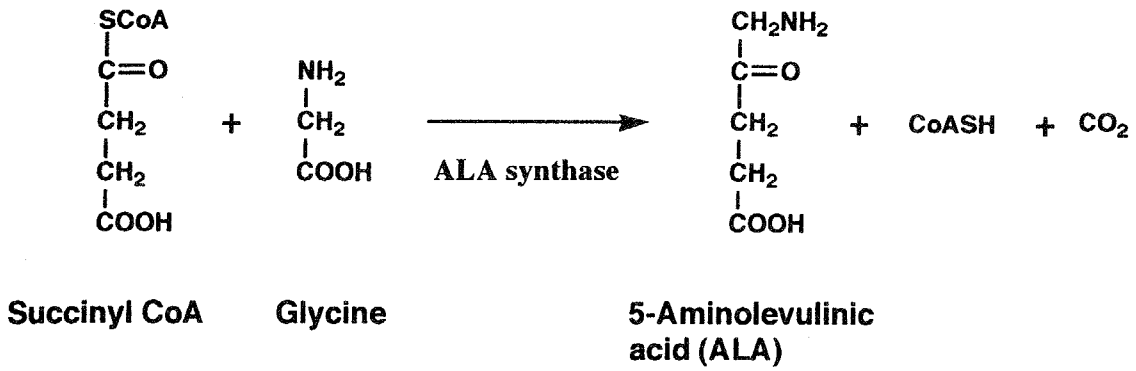
biosynthesis provides a major point for regulation of heme and Chl synthesis in prokaryotes, yeast, algae, plants, and in mammalian cells (Beale and Weinstein, 1990; Jordan, 1990).

The formation of tetrapyrroles from ALA is thought to occur by the same reactions in all species. In contrast, ALA formation occurs by two entirely different routes, which are confined to different groups of phototrophic species. The Shemin pathway (Shemin and Russell, 1953; Kikuchi *et al.*, 1958) involves the condensation of succinyl-coenzyme A and glycine catalyzed by the well characterized enzyme, ALA synthase (succinyl-CoA:glycine succinyltransferase [decarboxylating] EC 2.3.1.37; May *et al.*, 1986; Fig. I-2A). This pathway, originally described in the photosynthetic prokaryote, *Rhodobacter sphaeroides* and in avian cells, was subsequently shown to function in a limited number of eubacteria, yeast, and in mammalian cells (Beale and Weinstein, 1990; Jordan, 1990).

The second pathway of ALA biosynthesis, the C_5 pathway (Figure I-2B) was discovered in 1975 by radiotracer experiments that the 5-carbon skeleton of glutamate is transformed into ALA (Beale *et al.*, 1975) in plant chloroplasts. Lability of the ALA-forming activity in chloroplast extracts made us difficult to study the conversion of glutamate into ALA (Bruyant and Kannangara, 1987; Kannangara *et al.*, 1988). Wang *et al.* (1981) achieved the separation of three soluble enzymatic activities required for the *in vitro* transformation of glutamate into ALA and provided an evidence for the involvement of glutamate 1-semialdehyde (GSA) as the final intermediate (Kannangara and Gough, 1978; Houen *et al.*, 1983). The enzyme GSA aminotransferase was isolated from barley chloroplasts and shown to convert synthetic GSA into authentic ALA (Kannangara and Gough, 1978; Wang *et al.*, 1981).

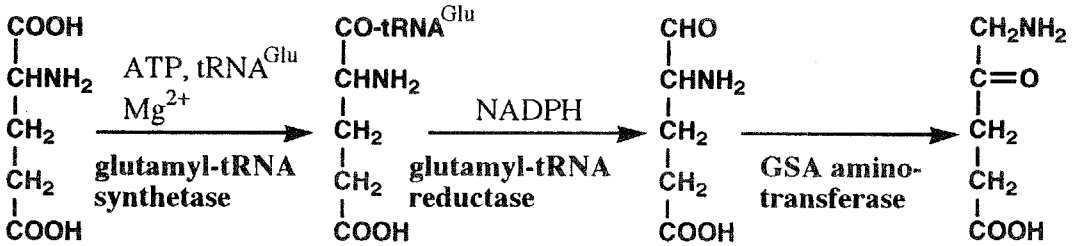
An RNase-sensitive component involved in the *in vitro* glutamate conversion to ALA was found in extracts from chloroplasts of barley and *Chlamydomonas reinhardtii* (Huang *et al.*, 1984; Kannangara *et al.*, 1984) and

(A)



Succinyl CoA Glycine 5-Aminolevulinic acid (ALA)

(B)



Glutamate Glutamyl-tRNA Glutamate 1-semialdehyde (GSA) 5-Aminolevulinic acid (ALA)

Figure I-2. Two biosynthetic pathways of 5-aminolevulinic acid (ALA). (A) Shemin's pathway and (B) C_5 pathway.

also from *Chlorella* (Weinstein and Beale, 1985a). Although the nature of the RNA and its role were unclear, it was proposed that the activation of glutamate is accomplished by a tRNA-like molecule (Huang *et al.*, 1984). Furthermore, it was shown that the RNA fraction active in ALA synthesis could be glutamylated. This suggested that the RNA responsible for ALA formation (δ-ALA RNA) was similar to $tRNA^{Glu}$ which can accept glutamate to form glutamyl-tRNA. NADPH-dependent reductase is responsible for the next reduction of this compound to GSA (Kannangara *et al.*, 1984). The tRNA nature of the δ-ALA RNA was established (Schön *et al.*, 1986) after purification of the active RNA species from the δ-ALA RNA fraction previously prepared from the barley chloroplasts (Kannangara *et al.*, 1984). Experiments by enzymatic removal of the 3'-terminal CCA and re-addition demonstrated the tRNA nature of the δ-ALA RNA (Schön *et al.*, 1986). Sequence analysis showed that a primary sequence could be folded into the typical cloverleaf structure of tRNAs. From the anticodon sequence UUC, the tRNA was identified as a $tRNA^{Glu}$, which was in agreement with the fact that the pure δ-ALA RNA could be charged with glutamate *in vitro* (Schön *et al.*, 1986). The tRNA nature of δ-ALA RNA was also shown by the fact that *Escherichia coli* $tRNA^{Glu}$ could be complement *Chlamydomonas* enzyme fraction in *in vitro* ALA formation (Huang and Wang, 1986). Hybridization of δ-ALA RNA fragments to nuclear and chloroplast DNA from barley showed that the RNA was encoded in the chloroplast genome. This was confirmed by sequence analysis of a chloroplast DNA fragment encoding this gene (Berry-Lowe, 1987). This work also showed that there was only one gene for $tRNA^{Glu}$ in the barley chloroplast genome (Berry-Lowe, 1987). Thus, the δ-ALA RNA is the normal $tRNA^{Glu}$ which is used for both ALA formation and protein synthesis.

Based on the information accumulated, a reaction mechanism for the C_5 pathway of ALA formation, which has served as the model mechanism to this date (Figure 1-2B), was proposed (Schön *et al.*, 1986; Kannangara *et al.*, 1988).

In the first step, glutamyl-tRNA synthetase attaches glutamate to tRNA, and generates Glu-tRNA^{Glu}. This reaction requires ATP and Mg^{2+}. The chloroplast enzyme, which is nuclear DNA-encoded, has been purified from wheat (Ratinaud *et al.*, 1983), barley (Bruyant and Kannangara, 1987) and *Chlamydomonas* (Chen *et al.*, 1990b). The next step involves the reduction of the activated glutamate by an NADPH-dependent reductase, glutamyl-tRNA reductase, to yield GSA. This enzyme has been purified from *Chlamydomonas* (Chen *et al.*, 1990a). In the final step, ALA is formed in a transamination catalyzed by GSA aminotransferase (Grimm *et al.*, 1989; Grimm, 1990). This enzyme has been purified from barley (Grimm *et al.*, 1989) and *Chlamydomonas* (Wang *et al.*, 1984). Its gene has been cloned from barley nuclear DNA (Grimm, 1990).

Studies in several laboratories have shown that the tRNA-dependent C_5 pathway of ALA synthesis exists in a wide variety of organisms, including eubacteria such as several species of photosynthetic bacteria and archaebacteria, *Chromatium vinosum*, *Methanobacterium thermoautotrophicum*, and in the chloroplasts of all algae and plant species tested so far (Huang *et al.*, 1984; Weinstein and Beale, 1985a,b; Friedmann and Thauer, 1986; Oh-hama *et al.*, 1986, 1988; Schneegurt and Beale, 1986; Weinstein *et al.*, 1986; Mayer *et al.*, 1987; Kannangara *et al.*, 1988; O'Neill *et al.*, 1989; Reible and Beale, 1988; Avissar and Beale, 1989; Li *et al.*, 1989).

In many organisms ALA formation is one of a key regulatory and rate-limiting step for the formation of hemes and Chls (May *et al.*, 1986; Beale and Weinstein, 1990; Jordan, 1990). The Chl pathway is thought to be regulated at three steps (Fig. I-1):

(a) at the conversion of protochlorophyllide to chlorophyllide *a*, which requires light in angiosperms and some algae, but not in some gymnosperms and most algae;

(b) at the branch point of metal insertion into Proto, leading to Chls upon magnesium insertion, and to heme in the case of iron incorporation; and
(c) at ALA formation, which is the earliest known step of the tetrapyrrole pathway.

In most cases studied, ALA formation has been found to be the rate-limiting step of Chl synthesis (May *et al.*, 1986; Beale and Weinstein, 1990; Jordan, 1990). Etiolated leaves contain all of the enzymes required for Chl synthesis from ALA. They are all present in the dark grown tissues, and no further enzyme synthesis is required for the Chl synthesis from ALA upon exposure to the light (Nadler and Granick, 1970; Schneider, 1973, 1976). In the dark, large amounts of protochlorophyllide accumulate when leaf tissues are incubated with ALA, and some of this protochlorophyllide is converted to Chl upon subsequent exposure of the tissues to the light (Castelfranco *et al.*, 1974; Sisler and Klein, 1963).

The synthesis of ALA is known to be strongly regulated by a number of factors, *i.e.*, light, plant hormones, and feedback inhibition by some intermediates. When completely dark-grown tissues are illuminated, Chl synthesis begins after a lag time of about 3 h. If the tissues are briefly preilluminated with white light and then placed back in the dark for a few hours, the Chl synthesis begins immediately upon reillumination. It is believed that the elimination of the lag phase is caused by the preillumination-triggered, dark-formation of the ALA-synthesizing system, which can then be become active immediately upon reillumination. Therefore, light could act as a crucial factor for the regulation of ALA synthesis. Actually, in tissues that normally require light for Chl synthesis, light is required for continuous formation of ALA.

Phytohormones are reported as another regulator of the Chl synthesis. Pretreatment of etiolated wheat leaves (Beevers *et al.*, 1970) or cucumber cotyledons (Fletcher and McCullagh, 1971) with cytokinins abolishes the

subsequent lag phase during light-induced greening period. In sunflower cotyledons, benzyladenine (BA), one of cytokinins, had no effect on the steady state rate of the Chl synthesis achieved after the end of the lag phase, but BA greatly shortened the initial lag phase (Ford *et al.*, 1979). It appears that exogenous cytokinins stimulate the formation of the ALA-synthesizing system, so that after exposure to the light, the Chl formation can proceed immediately without the lag where the ALA-synthesizing system is made *de novo*.

The synthesis of ALA is also controled through a feedback regulation by its intermediates and end products. Heme is the most potent inhibitor of this step. Heme may play a significant role in regulating ALA formation from glutamate by affecting enzyme activity (Weinstein and Beale, 1985b).

Thus, it is concluded that the regulation of ALA formation is central to the coordination of the Chl synthesis and the development of photosynthetic competence in plants. However, the mechanism of regulation of ALA synthesis is not fully understood in higher plants. In the present study, I investigated the mechanism of regulation of ALA synthesis. First, in order to examine the expression of $tRNA^{Glu}$, I isolated the cucumber chloroplast gene encoding $tRNA^{Glu}$ which is involved in ALA synthesis, and determined the sequence of this gene. This is discussed in Chapter II. Second, effects of light and cytokinin on ALA synthesizing components are described in Chapter III. Thirdly, mechanism of feedback regulation of ALA synthesis was examined by elucidating the mode of action of the specific inhibitor of the Chl biosynthetic pathway, as discussed in Chapter IV. Finally, in Chapter V, a model for mechanism of regulation of ALA synthesis in cucumber is proposed.

REFERENCES

Avissar, Y. J. and Beale, S. I. (1989) Distribution of δ-aminolevulinic acid biosynthetic pathways among phototrophic bacterial groups. *Arch. Microbiol.*, **151**: 513-519.

Beale, S. I. and Weinstein, J. D. (1990) Tetrapyrrole metabolism in photosynthetic organisms. In Dailey, H. A. (ed.), *Biosynthesis of Heme and Chlorophylls*. McGraw-Hill, New York, pp. 287-391.

Beale, S. I., Gough, S. P. and Granick, S. (1975) Biosynthesis of δ-aminolevulinic acid from the intact carbon skeleton of glutamic acid in greening barley. *Proc. Natl. Acad. Sci. USA*, **72**: 2719-2723.

Beevers, L., Lovey, B., Pearson, J. A. and Wareing, P. F. (1970) Phytochrome and hormonal control of expansion and greening of etiolated wheat leaves. *Planta*, **90**: 286-294.

Berry-Lowe, S. (1987) The chloroplast glutamate tRNA gene required for δ-aminolevulinate synthesis. *Carlsberg Res. Commun.*, **52**: 197-210.

Bruyant, P. and Kannangara, C. G. (1987) Biosynthesis of δ-aminolevulinate in greening barely leaves. VIII. Purification and characterization of the glutamate-tRNA ligase. *Carlsberg Res. Commun.*, **52**: 99-109.

Castelfranco, P. A., Rich, P. M. and Beale, S. I. (1974) The abolition of the lag phase in greening cucumber cotyledons by δ-aminolevulinic acid. *Plant Physiol.*, **53**: 615-618.

Castelfranco, P. A. and Beale, S. I, (1983) Chlorophyll biosynthesis: recent advances and areas of current interest. *Annu. Rev. Plant Physiol.*, **34**: 241-278.

Chen, M. W., Jahn, D., O'Neill, G. P. and Söll, D. (1990a) Purification of the glutamyl-tRNA reductase from *Chlamydomonas reinhardtii* involved in δ-aminolevulinic acid formation during chlorophyll biosynthesis. *J. Biol. Chem.*, **265**: 4058-4063.

Chen, M. W., Jahn, D., O'Neill, G. P. and Söll, D. (1990b) Purification and characterization of *Chlamydomonas reinhardtii* chloroplast glutamyl-tRNA synthetase, a natural misacylating enzyme. *J. Biol. Chem.*, **265**: 4054-4057.

Fletcher, R. A. and McCullagh, D. (1971) Benzyladenine as a regulator of chlorophyll synthesis in cucumber cotyledons. *Can. J. Bot.*, **49**: 2197-2201.

Ford, M. J., Alhadeff, M., Chapman, J. M. and Black, M. (1979) A rapid and selective action of 6-benzylaminopurine on 5-aminolevulinate production in excised sunflower cotyledons. *Plant Sci. Lett.*, **16**: 397-402.

Friedmann, H. C. and Thauer, R. K. (1986) Ribonuclease-sensitive δ–aminolevulinic acid formation from glutamate in cell extracts of *Methanobacterium thermoautotrophicum*. *FEBS Lett.*, **207**; 84-88.

Grimm, B. (1990) Primary structure of a key enzyme in plant tetrapyrrole synthesis: glutamate 1-semialdehyde aminotransferase. *Proc. Natl. Acad. Sci. USA*, **87**: 4169-4173.

Grimm, B., Bull, A., Welinder, K. G., Gough, S. P. and Kannangara, C. G. (1989) Purification and partial amino acid sequence of the glutamate 1-semialdehyde aminotransferase of barley and *Synechococcus*. *Carlsberg Res. Commun.*, **54**: 67-79.

Houen, G., Gough, S. P. and Kannangara, C. G. (1983) δ–Aminolevulinate synthesis in greening barley V. The structure of glutamate 1-semialdehyde. *Calrsberg Res. Commun.*, **48**: 567-572.

Huang, D. D. and Wang, W. Y. (1986) Chlorophyll biosynthesis in *Chlamydomonas* starts with the formation of glutamyl-tRNA. *J. Biol. Chem.*, **261**: 13451-13455.

Huang, D. D., Wang, W. Y., Gough, S. P. and Kannangara, C. G. (1984) δ–Aminolevulinic acid-synthesizing enzymes need an RNA moiety for activity. *Science*, **225**: 1482-1484.

Jordan, P. M. (1990) Biosynthesis of 5-aminolevulinic acid and its transformation into coproporphyrinogen in animal and bacteria. In Dailey, H. A. (ed.), *Biosynthesis of Heme and Chlorophylls*. McGraw-Hill, New York, pp. 55-212.

Kannangara, C. G. and Gough, S. P. (1978) Biosynthesis of δ-aminolevulinate in greening barley leaves: glutamate 1-semialdehyde aminotransferase. *Carlsberg Res. Commun.*, **43**: 185-194.

Kannangara, C. G., Gough, S. P., Oliver, R. P. and Rasmussen, S. K. (1984) Biosynthesis of δ-aminolevulinate in greening barley leaves. VI. Activation of glutamate by ligation to RNA. *Carlsberg Res. Commun.*, **49**: 417-437.

Kannangara, C. G., Gough, S. P., Bruyant, P., Hoober, J. K., Kahn, A. and von Wettstein, D. (1988) $tRNA^{Glu}$ as a cofactor in δ-aminolevulinate biosynthesis: steps that regulate chlorophyll synthesis. *Trends Biochem. Sci.*, **13**: 139-143.

Kikuchi, G., Kumar, A., Talmage, P. and Shemin, D. (1958) The enzymatic synthesis of 5-aminolevulinic acid. *J. Biol. Chem.*, **233**: 1214-1219.

Li, J. M., Brathwaite, O., Cosloy, S. D. and Eussell, C. S. (1989) 5-Aminolevulinic acid synthesis in *Escherichia coli*. *J. Bacteriol.*, **171**: 2547-2552.

May, B. K., Borthwick, I.A., Srivastava, G., Pirola, B. A. and Elliott, W. H. (1986) Control of 5-aminolevulinate synthase in animals. *Current Topics Cellular Reg.*, **28**: 233-262.

Mayer, S. M., Beale, S. I. and Weinstein, J. D. (1987) Enzymatic conversion of glutamate to δ-aminolevulinic acid in soluble extracts of *Euglena gracilis*. *J. Biol. Chem.*, **262**: 12541-12549.

Nadler, K. and Granick, S. (1970) Controls on chlorophyll synthesis in barley. *Plant Physiol.*, **46**: 240-246.

Oh-hama, T., Seto, H. and Miyachi, S. (1986) ^{13}C-NMR evidence for bacteriochlorophyll *a* formation by the C_5 pathway in *Chromatium*. *Arch. Biochem. Biophys.*, **246**: 192-198.

Oh-hama, T., Stolowich, N. J. and Scott, A. I. (1988) 5-Aminolevulinic acid formation from glutamate via the C_5-pathway in *Clostridium thermoaceticum*. *FEBS Lett.*, **228**: 89-93.

O'Neill, G. P., Peterson, D. M., Schön, A., Chen, M. W. and Söll, D. (1989) δ–Aminolevulinic acid biosynthesis in *Escherichia coli* and *Bacillus subtillis* involves formation of glutamyl-tRNA. *FEMS Microbiol. Lett.*, **60**: 255-260.

Ratinaud, M. H., Thomes, J. C., and Julien, R. (1983) Glutamyl-tRNA synthetases from wheat. Isolation and characterization of three dimeric enzymes. *Eur. J. Biochem.*, **135**: 471-477.

Rieble, S. and Beale, S. I. (1988) Transformation of glutamate to δ–aminolevulinic acid by soluble extracts of *Synechocystis* sp. PCC 6803 and other oxygenic prokaryotes. *J. Biol. Chem.*, **263**: 8864-8871.

Schneegurt, M. A. and Beale, S. I. (1986) Biosynthesis of protoheme and heme a precursors from glutamate in maize. *Plant Physiol.*, **81**: 965-971.

Schneider, H. A. W. (1973) Regulation der chlorophyllbiosynthese. Licht und entwicklungsbedingte Aktivitätsänderungen von vier aufeinander folgenden Enzymen der Porphyrin- und Chlorophyllbiosynthesekette. *Z. Naturforsch.*, **28C**: 45-58.

Schneider, H. A. W. (1976) Enzymatic capacities for chlorophyll synthesis. Activation and *de novo* synthesis of enzymes. *Z. Naturforsch.*, **31C**: 55-63.

Schön, A., Krupp, G., Gough, S. P., Berry-Lowe, S., Kannangara, C. G. and Söll, D. (1986) The RNA required in the first step of chlorophyll biosynthesis is a chloroplast glutamate tRNA. *Nature*, **331**: 187-190.

Shemin, D. and Russell, C. S. (1953) 5-Aminolevulinic acid, its role in the biosynthesis of porphyrins and purines. *J. Am. Chem. Soc.*, **75**: 4873-4874.

Sisler, E. C. and Klein, W. H. (1963) The effect of age and various chemicals on the lag phase of chlorophyll synthesis in dark grown bean seedlings. *Plant Physiol.*, **16**: 315-322.

Wang, W. Y., Gough, S. P. and Kannangara, C. G. (1981) Biosynthesis of δ–aminolevulinate in greening barley leaves IV. Isolation of three soluble enzymes required for the conversion of glutamate to δ–aminolevulinate. *Carlsberg Res. Commun.*, **46**: 243-257.

Wang, W. Y., Huang, D. D., Stachon, D., Gough, S. P. and Kannangara, C. G. (1984) Purification, characterization, and fractionation of the δ–aminolevulinic acid synthesizing enzymes from light-grown *Chlamydomonas reinhardtii* cells. *Plant Physiol.*, **74**: 569-575.

Weinstein, J. D. and Beale, S. I. (1985a) RNA is required for enzymatic conversion of glutamate to δ–aminolevulinate by extracts of *Chlorella vulgaris*. *Arch. Biochem. Biophys.*, **239**: 87-93.

Weinstein, J. D. and Beale, S. I. (1985b) Enzymatic conversion of glutamate to δ–aminolevulinate in soluble extracts of the unicellular alga *Chlorella vulgaris*. *Arch. Biochem. Biophys.*, **237**: 454-464.

Weinstein, J. D., Mayer, S. M. and Beale, S. I. (1986) Stimulation of δ–aminolevulinic acid formation in algal extracts by heterologous RNA. *Plant Physiol.*, **82**: 1096-1101.

Chapter II

Structure of $tRNA^{Glu}$ Gene of Cucumber Chloroplast Genome

INTRODUCTION

Involvement of $tRNA^{Glu}$ in the ALA synthesis has been demonstrated in a number of organisms such as barley and *Chlamydomonas reinhardtii.* (Huang *et al.*, 1984; Weinstein *et al.*, 1986; Kannangara *et al.*, 1988). The $tRNA^{Glu}$ is encoded in the chloroplast genome. In barley, the active $tRNA^{Glu}$ fraction has been isolated by the chlorophyllin- or heme-Sepharose column chromatography (Kannangara *et al.*, 1984). There is only one gene for $tRNA^{Glu}$ in the barley chloroplast genome, and cotranscribed with $tRNA^{Tyr}$ and $tRNA^{Asp}$ (Berry-Lowe, 1987). Since a single $tRNA^{Glu}$ is used for the synthesis of both ALA and proteins, there may be a regulatory mechanism to coordinate the rates of these two pathways during greening.

ALA synthesis, the limiting step in chlorophyll biosynthesis, is regulated not only by light but also by plant hormones (Chapter III). Although substantial works on the chloroplast biogenesis have been done for a number of monocot species, they are less sensitive to plant hormones than dicots in their response that the greening is enhanced. A dicot, cucumber, is widely used to study the chloroplast development and chlorophyll biosynthesis. It has been shown that in cucumber a cytokinin, benzyladenine (BA), stimulates ALA synthesis and protochlorophyllide regeneration and eliminates the lag phase for chlorophyll accumulation (Lew and Tsuji, 1982). Therefore, studying the ALA synthesizing system using cucumber is significant for the understanding of the

regulation of ALA synthesis by light and hormones. However, there has been little information about the involvement of $tRNA^{Glu}$ in ALA synthesis in dicots. With cucumber, the ALA synthesis has been demonstrated *in vivo* and in plastids, but the ALA synthesis by plastid extracts has not been characterized *in vitro*, because the plastid extracts are very labile (Kannangara *et al.*, 1988). The sequence of chloroplast (plastids) $tRNA^{Glu}$ gene has been reported for tobacco, spinach and pea (Holschuh *et al.*, 1984; Rasmussen *et al.*, 1984; Ohme *et al.*, 1986), but nothing is known about the cucumber gene.

In this chapter, I described the isolation of RNA fraction from greening cucumber cotyledons, and showed that this fraction contained $tRNA^{Glu}$ and stimulated ALA synthesis *in vitro*. I isolated the cucumber chloroplast gene encoding $tRNA^{Glu}$ and determined the sequence of the gene. The obtained clone was used for the assay of the expression of this gene in Chapter III.

MATERIALS AND METHODS

Plant material and growth conditions

Cucumber (*Cucumis sativus* L. cv. Aonagajibai) seeds were germinated on wet vermiculite for 4 days in the dark at 28°C. Cotyledons were exposed to fluorescent light (Mitsubishi FLR 80H W/A, Mitsubishi Electric Co., Tokyo) for 6 h at an intensity of 43 μmol/m^2 s.

Isolation of plastids and stromal proteins

The isolation of plastids was carried out at 0-4°C according to the procedure of Kannangara and Gough (1977) with a slight modification (Dei and Tsuji 1987). After 6 h of illumination, cotyledons were harvested and those of 100 g fresh weight were homogenized in 300 ml of chilled grinding medium (0.5 M glycerol, 0.1 M Tricine (pH 8.0), 1 mM DTT, 1 mM $MgCl_2$, 1 mM EDTA) using a precooled modified kitchen homogenizer (Hitachi VA-76G, Hitachi Electric Co., Tokyo) equipped with razor blades. The homogenate was filtered through two layers of Miracloth and the filtrate was centrifuged at 100 x*g* for 2 min, followed by another centrifugation of the supernatant at 1,500 x*g* for 8 min. The plastid pellets were resuspended in a small volume of column buffer (0.3 M glycerol, 0.1 M Tricine (pH 8.0), 1 mM DTT, 25 mM $MgCl_2$, 1 mM EDTA) and then disrupted by Polytron-type homogenizer for 1 min. The disrupted plastids were centrifuged at 300,000 x*g* for 90 min. The resultant supernatant (stromal proteins) was used for the following analysis.

Preparation of chlorophyllin-Sepharose

Chlorophyllin-Sepharose was prepared as described by Kannangara *et al.* (1984). Na-chlorophyllin (270 mg) was dissolved in 200 ml of water and the pH was adjusted to 9.5 with NaOH. EAH-Sepharose (Pharmacia, Uppsala, Sweden; 7.5 g) was swollen in 300 ml of 0.5 M NaCl and washed with 1,500 ml

of 0.5 M NaCl followed by 1,500 ml distilled water. The chlorophyllin fraction was mixed with the gel suspension. *N*-Ethyl-*N*'-(dimethylaminopropyl)-carbodiimide hydrochloride (57 mg) dissolved in 1 ml of water was added. The pH dropped slowly and was adjusted to 8.0 during first 15 min. The suspension was covered with aluminum foil and gently shaken for 2 days at 21°C. The gel was then filtered. To block any residual amino groups, gels were suspended in 10% acetic acid, and then 1 ml of water containing 57 mg of carbodiimide was added and the suspension was shaken overnight. The chlorophyllin-Sepharose was washed with water. Non-covalently bound chlorophyllin was then removed by washing alternately with a solution of 0.5 M NaCl containing 0.1 M Na-acetate adjusted to pH 4.5 with acetic acid and a solution of 0.5 M NaOH containing 7 M urea. The gel was stored in the acetate buffer and was equilibrated with the column buffer (0.3 M glycerol, 0.1 M Tricine ,pH 8.0, 1 mM DTT, 25 mM $MgCl_2$, 1 mM EDTA).

Gel filtration and affinity chromatography of stroma proteins

Gel filtration and affinity chromatography were carried out as Wang *et al.* (1981) using columns of Sepharose CL-6B and chlorophyllin-Sepharose. The column used had dimensions in cm: Sepharose CL-6B 3 × 30 and chlorophyllin-Sepharose 1.5 × 20. Both columns were equilibrated with column buffer as mentioned above, and the bound fraction was eluted from the chlorophyllin-Sepharose column with 1 M NaCl dissolved in the same medium.

SDS-polyacrylamide gel electrophoresis and Western blot analysis

The protein fractions eluted from Sepharose CL-6B were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was carried out according to Laemmli (1970) with stacking and separation gels of 5% and 14% polyacrylamide, respectively. After electrophoresis, the gels were stained with Comassie Brilliant Blue R-250 or electroblotted onto nitrocellulose

filter for Western blot analysis. Anti-barley glutamate 1-aminotransferase antibodies cross-reacted with the cucumber enzyme. Antigen-antibody complexes were visualized by using alkaline phosphatase (Blake *et al.* 1984).

Isolation of chlorophyllin-Sepharose-bound RNA by phenol extraction and ethanol precipitation

The fraction eluted from the chlorophyllin-Sepharose column was mixed with an equal volume of phenol saturated with 0.1 M Tris-HCl (pH 8.0) and 2 mM EDTA. The mixture was shaken for 10 min in cold room and centrifuged for 5 min. The lower phenol phase was discarded and the phenolisation procedure was repeated. Phenol was removed from the aqueous phase by washing with chloroform:isoamylalcohol (24:1). One-tenth volume of 3 M Na-acetate (pH 5.5) and 2.5 volumes of ethanol were added and the solution was kept at -80°C for 1 h to precipitate the RNA. The RNA was collected by centrifugation at 3,000 rpm for 10 min. RNA pellets were rinsed with 70% ethanol and dried in a vacuum desiccator and dissolved in 100 μl of distilled water.

Assay for ALA synthesizing activity

Assay for ALA synthesizing activity was carried out by the method of Dei and Tsuji (1987). Incubation was done for 60 min at 29°C in 1 ml of reaction medium containing 0.3 M glycerol, 0.1 M Tricine ,pH 8.0, 1 mM EDTA, 25 mM $MgCl_2$, 1 mM L-glutamate, 5 mM ATP, 1.5 mM NADPH, 10 mM levulinic acid (LA), 1-6 mg of nonbound enzyme of chlorophyllin-Sepharose, and 1 A_{260} unit of the chlorophyllin-Sepharose-bound RNA. Reactions were terminated by addition of 100 μl of TCA. ALA was isolated on Dowex 50W-X8 (Kannangara *et al.*, 1984). The pH of the eluate was adjusted to 4.6, and an aliquot (1.5 ml) of the eluate was heated to 100°C for 10 min with 50 μl of acetylacetone (Mauzerall and Granick, 1956). After cooling, equal volumes of the sample and

Ehrlich-Hg reagent were mixed (Urata and Granick, 1963). Fifteen minutes later, absorbance at 553 nm (A_{553}) of the mixture was read in a Hitachi 556 spectrophotometer against a control that had been treated identically except that acetylacetone was replaced by water. The molar extinction coefficient of 7 x 10^4 ALA was employed (Mauzerall and Granick, 1956).

Chloroplast DNA isolation

Cucumber seedlings were grown under the fluorescent light at 28°C as described before. At about 4 days of germination, cotyledons were placed in the dark for 1 day to be starved of polysaccharide or starch, which disturbs the purification of DNA. Plastids were isolated as mentioned above in an ice-chilled homogenization buffer (50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 0.6 M mannitol). The plastid pellets were resuspended in the same buffer and clumps were broken up by a few gentle strokes in a glass homogenizer. The suspension was centrifuged and the procedure was repeated twice. To remove large particles such as cell debris and nuclei, the plastid suspension was then subjected to step-wise sucrose density gradient (15 ml of 40%, and 20 ml of 20% sucrose in homogenization buffer minus 0.6 M mannitol) and centrifuged at 800 x*g* for 20 min. The plastid band between 20% and 40% layer was removed and pelleted by centrifugation at 8,000 x*g* for 15 min. Chloroplasts were lysed in the same buffer omitting mannitol and the DNA purified by centrifugation through cesium chloride cushions for 48 h at 100,000 x*g* according to Ohyama *et al.* (1982).

Oligonucleotide synthesis and labeling

Two oligonucleotides were synthesized on an Applied Biosystems Oligonucleotide Synthesizer. One oligonucleotide probe (39 nucleotides) is complementary to the $tRNA^{Glu}$ sequence of barley chloroplast genome and was used for hybridization to identify $tRNA^{Glu}$ involved in ALA synthesis to screen

cucumber chloroplast genomic library and for Northern blot analysis. The other (40 nucleotides) is complementary to the $tRNA^{Phe}$ sequence of barley chloroplast genome and was used for control of Northern blot analysis. Both oligonucleotides were 5' end-labelled with [α-^{32}P]ATP (> 7000 Ci/mmol) by T4 polynucleotide kinase (Takara Biomedicals Co., Ltd, Kyoto) .

Southern blot analysis

Restriction fragments obtained by digestion of cucumber chloroplast DNA were separated by electrophoresis through 0.7% agarose gels and transferred to a nylon membrane (Maniatis *et al.*, 1982). The blots were screened with the ^{32}P-labelled oligonucleotide complementary to $tRNA^{Glu}$ sequence. The membrane was hybridized for 18 h at 60°C in blotting buffer. The blotting buffer was prepared by dissolving Hybridization buffer tablets (Amasham) in 10 ml of distilled water. To remove nonspecifically bound probe, the membrane was washed in 2 × SSC containing 0.1% SDS two times for 15 min each at 23°C and in 0.2 × SSC containing 0.1% SDS two times for 30 min each at 23°C.

Northern blot analysis

Ten μg of chlorophyllin-Sepharose bound RNA were fractionated by electrophoresis on 10% polyacrylamide-8 M urea gels and electroblotted to nylon membranes according to the manufacturer with 20 mM Tris-HCl (pH 7.2), 2.5 mM Na acetate and 1 mM EDTA as the transfer buffer. The condition of hybridization was same as described in Southern blot analysis. In this case, hybridization was analyzed by Bio-image Analyzer BA 100A (Fuji film, Co. Ltd, Tokyo).

Cloning of the cucumber chloroplast $tRNA^{Glu}$ gene (*trnE*)

Cucumber chloroplast DNA was digested to completion with Hind III and the restriction fragments were separated by electrophoresis on 0.7% LMP

agarose (BRL Life Technologies Inc.). DNA fragments were isolated from the gel by melting at 65°C and extracted with phenol. The recovered DNA fragments were ligated into the Hind III site of the vector pUC119. The ligation reaction mix was transformed into *E. coli* MV1190, and the clones containing recombinant plasmids were transferred to a nylon membrane and screened by colony hybridization with the probe. The condition of hybridization is the same as described in Southern blot analysis.

DNA sequencing

Deletion mutants of plasmid containing 1.3-kbp insert were constructed by unidirectional exonuclease III digestion (Henikoff, 1984). Plasmid DNA preparations were made by the alkali method (Birnboin and Doly, 1979). Double-stranded, supercoiled plasmid DNAs were sequenced by the dideoxy chain-termination method with [α-^{32}P]dCTP and the Sequenase sequencing kit (United States Biochemical Corp., Cleveland, Ohio) according to the instructions of the manufacturer.

RESULTS

Isolation of chlorophyllin-Sepharose bound RNA

Stromal proteins obtained by high speed centrifugation were fractionated by Sepharose CL-6B gel filtration. Three main ultraviolet light absorbing peaks were eluted from the column (Fig. II-1A). The second peak contained soluble proteins (Fig. II-1B) cross-reacting with antiserum against the barley glutamate 1-semialdehyde aminotransferase (Fig. II-1C) (Grimm *et al.*, 1989). The fractions were collected and subjected to chlorophyllin-Sepharose chromatography. Bound substances were eluted with the column medium containing 1 M NaCl (Fig. II-2). The fractions showed the absorption spectrum with a maximum at 260 nm and a 260/280 ratio of 1.86 characteristic of nucleic acid. The run-off fractions alone did not stimulate ALA synthesis but did when combined with the bound fraction (Table II-1). RNA in the bound fraction was extracted by the phenol method, precipitated with ethanol, and then separated by electrophoresis on a polyacrylamide gel containing urea (Fig. II-3A). Northern blot analysis showed that two bands were hybridized with the oligonucleotide probe complementary to the $tRNA^{Glu}$ gene of the barley chloroplast (5'-AGTCGAATCCCCGCTGCCTCCTTGAAAGAGAGATGTCCT-3') (Fig. II-3B). However, the lower one could be considered as degradation products of the tRNA since it was broad and of low molecular weight. Together, these results suggest that the chlorophyllin-Sepharose-bound fraction from cucumber cotyledons contains the $tRNA^{Glu}$ which participates in ALA synthesis as reported for other plant species (Huang *et al.*, 1984; Weinstein *et al.*, 1986; Kannangara *et al.*, 1988).

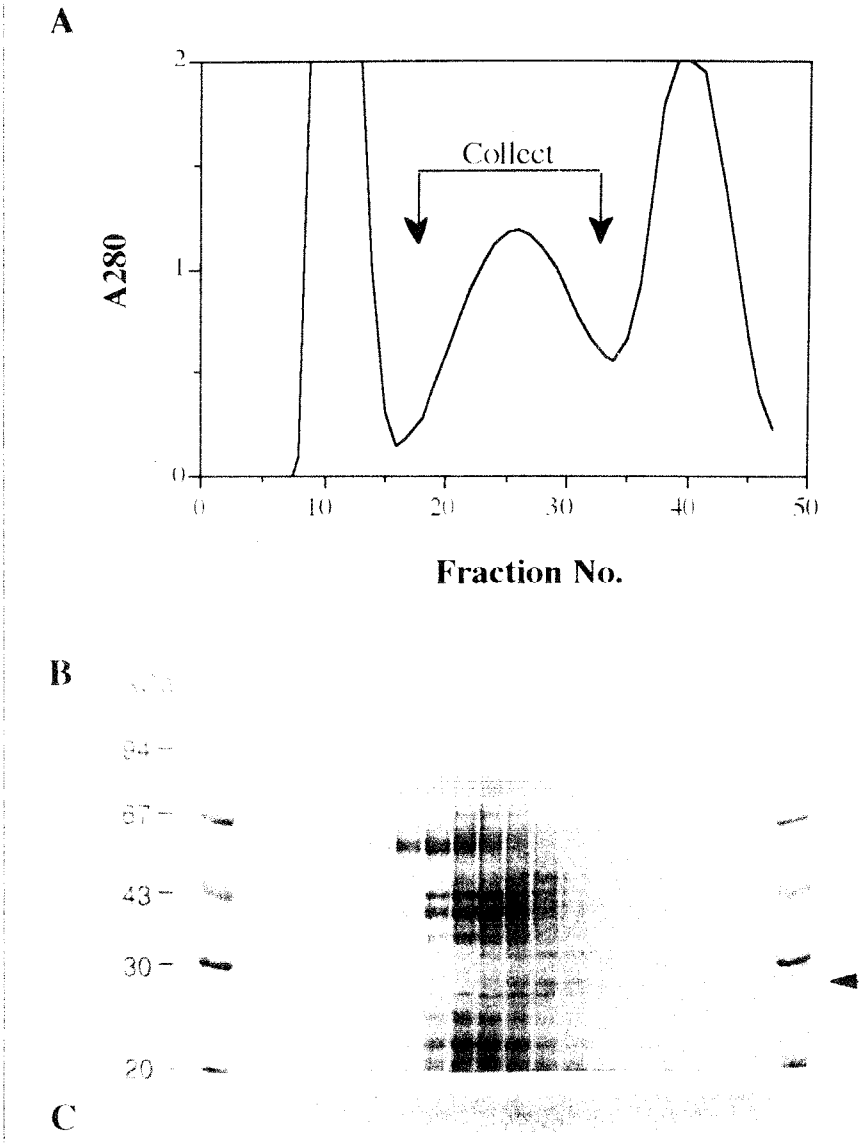


Figure II-1. Fractionation of stromal proteins by Sepharose CL-6B column chromatography. A, elution profile of stromal proteins from Sepharose CL-6B; B, ten μl of each fraction was separated on SDS-PAGE. Gels were stained with Coomassie Brilliant Blue; C, gels were electroblotted onto nitrocellulose filter, and immunoblotted with an antiserum against barley glutamate 1-semialdehyde aminotransferase. In panel B, the arrow head indicates the band corresponding to a molecular mass of 26 kDa which is identified by the antibody.

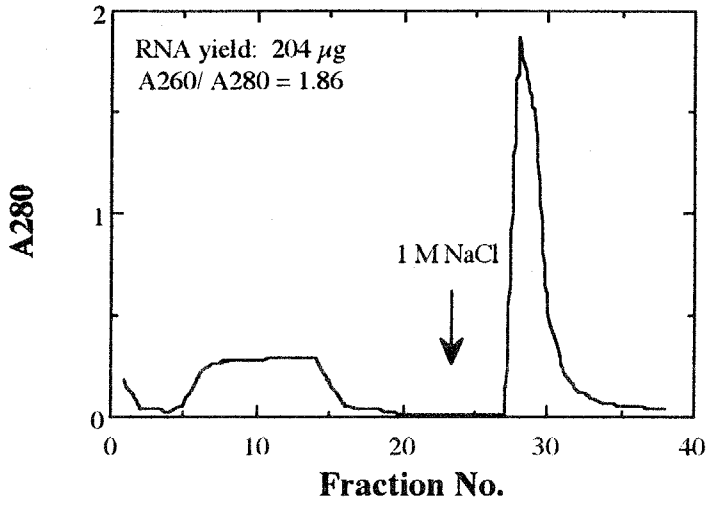


Figure II-2. Elution profile of chlorophyllin-Sepharose chromatography. Combined fractions from Sepharose CL-6B gel filtration were loaded onto chlorophyllin-Sepharose column. Bound fractions were eluted with the column buffer containing 1 M NaCl. The A_{260}/A_{280} ratio and recovery of RNA are indicated in the figure.

Table II-1

Reconstitution Experiments with the Run-Off and Bound Fractions of Chlorophyllin-Sepharose Column Chromatography.

Incubation mixture	ALA formed (nmol/mg protein·h)
1, Crude enzyme fraction from Sepharose CL-6B chromatography	186
2, Run-off fraction	70
3, Run-off + Chlorophyllin bound fraction	133

Experimental conditions: 1, two mg protein of crude enzyme fraction obtained from Sepharose CL-6B gel filtration; 2, two mg protein of the run-off fraction of chlorophyllin-Sepharose chromatography; 3, two mg protein of the run-off fraction and the bound fraction containing 40 μg RNA of chlorophyllin-Sepharose chromatography.

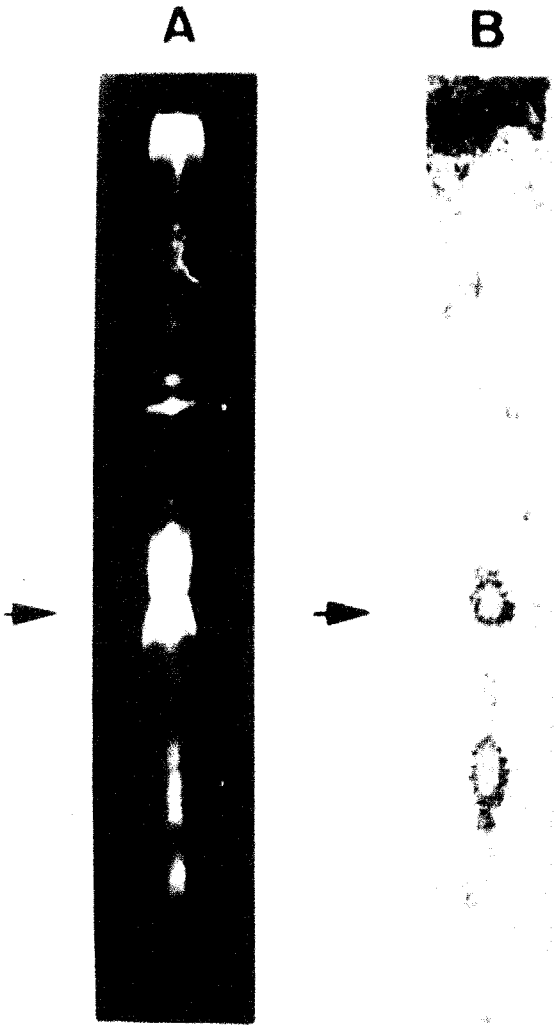


Figure II-3. Separation of chlorophyllin-bound RNA by urea-polyacrylamide gel electrophoresis and Northern hybridization with a $tRNA^{Glu}$ probe. Ten μg of chlorophyllin-bound RNA was electrophoresed on urea-polyacrylamide gel. A, ethidium bromide stained gel; B, the RNA was transferred to a nylon membrane and hybridized with the synthetic oligonucleotide probe for $tRNA^{Glu}$, followed by monitoring with an image analyzer.

Cloning of $tRNA^{Glu}$ gene

Using the same oligonucleotide probe, cucumber chloroplast genomic library was screened for genes coding for $tRNA^{Glu}$. A single band of hybridization was detected when the end-labelled oligonucleotide was hybridized with chloroplast DNA digested with *Eco*RI, *Bam*HI or *Hin*dIII (Fig. II-4). In either case, a minor band observed just above the major one disappeared when the membrane was washed extensively, suggesting that it may be an artifact. The 3.9 kb *Hin*dIII fragment was cloned into pUC119 plasmid vector to generate clone pTE3.9. Digestion of pTE3.9 with *Hin*dIII and *Xba*I gave two fragments of 1.3 kb and 2.6 kb. Since only the former hybridized with the probe (data not shown), it was subcloned into pUC119 to generate pTE1.3. Fine structure and sequencing strategy of the 1.3-kb *Hin*dIII-*Xba*I insert are shown in Figure II-5. The 1.3-kb fragment was deleted by exonuclease III and exonuclease VII, and subcloned for sequencing. The reverse strand was also sequenced.

Sequence of $tRNA^{Glu}$ gene

Figure II-6 shows the complete nucleotide sequence of the 1.3-kb fragment of pTE1.3. Comparison of this sequence with tRNA sequences revealed that the 1.3-kb fragment encoded two tRNA genes for glutamate (*trnE*, UUC) and threonine (*trnT*, GGU). Figure II-7 shows the DNA sequence for $tRNA^{Glu}$ cloverleaf structure. The *trnE* was 73 nucleotides long, and had an A:U base pair in position 53:61 (arrows in Fig. II-7). The *trnT* gene was encoded on the reverse strand to *trnE* gene.

Comparison of sequences with other species

The cucumber chloroplast *trnE* gene showed strong homology to those of other species (Fig. II-8). It was more than 95% homologous to those of barley, tobacco, spinach and pea, and more than 85% homologous to that of

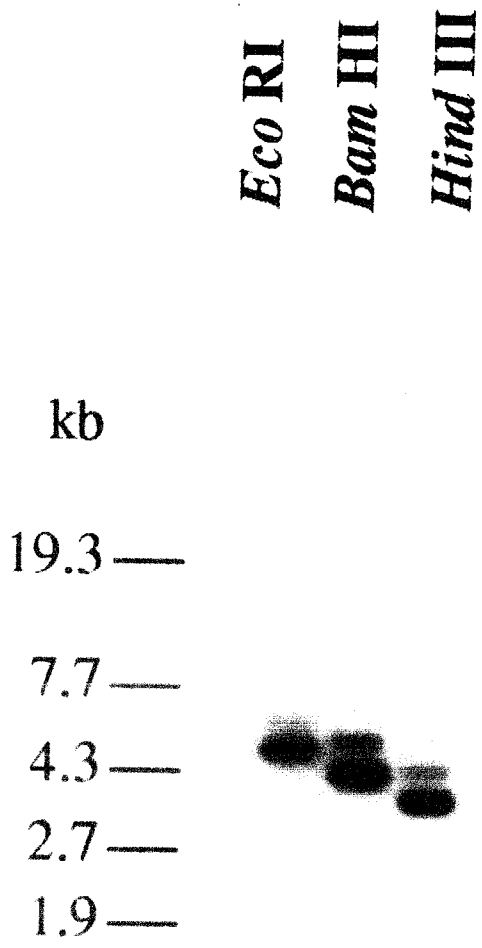


Figure II-4. Autoradiogram of hybridization of the cucumber chloroplast genome with a $tRNA^{Glu}$ probe. Five micrograms of purified chloroplast DNA was digested with *Eco*RI, *Bam*HI or *Hind*III, electrophoresed on a 0.7% agarose gel, transferred to nylon membrane and hybridized with the $tRNA^{Glu}$ probe. Lambda DNA digested with *Sty*I was used as size markers which are shown in kb on the left side.

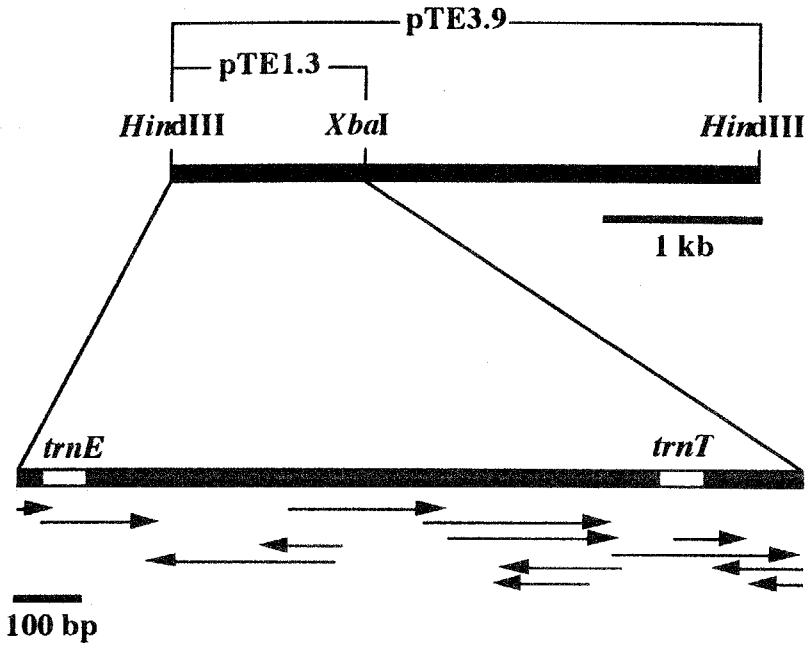


Figure II-5. Physical map and sequencing strategy of the 1.3-kb *Hin*dIII-*Xba*I subclone. Arrows indicate the origin and extent of DNA sequences. The locations of the tRNA genes for glutamate (*trnE*) and threonine (*trnT*) are shown by white boxes.

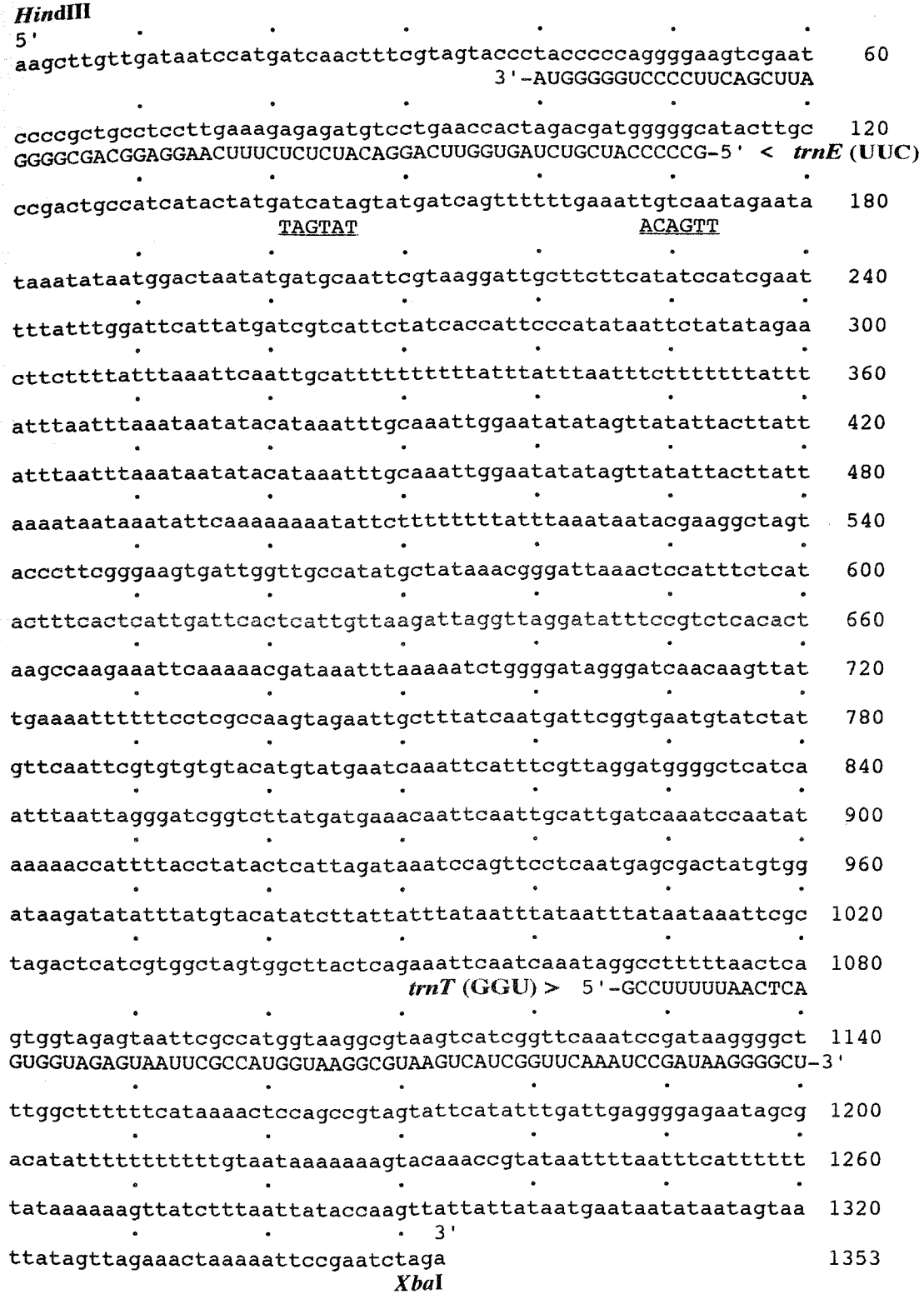


Fig. II-6. Complete nucleotide sequence of the cucumber chloroplast genome clone pTE 1.3. The RNA transcripts for *trnE* and *trnT* are given under the DNA sequence by capitals. The arrow heads with the gene name indicate the 5' end of its coding sequence and direction of transcription. Putative promoter sequences of *trnE* are indicated by capitals with underline.

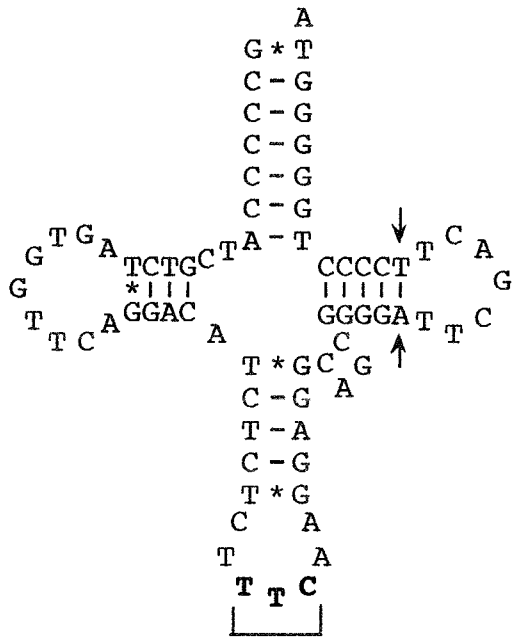


Figure II-7. DNA sequence for cucumber chloroplast $tRNA^{Glu}$. Arrows indicate an A:U base pair in position 53:61 which is replaced by G:C base pair in all tRNAs coding for other amino acids.

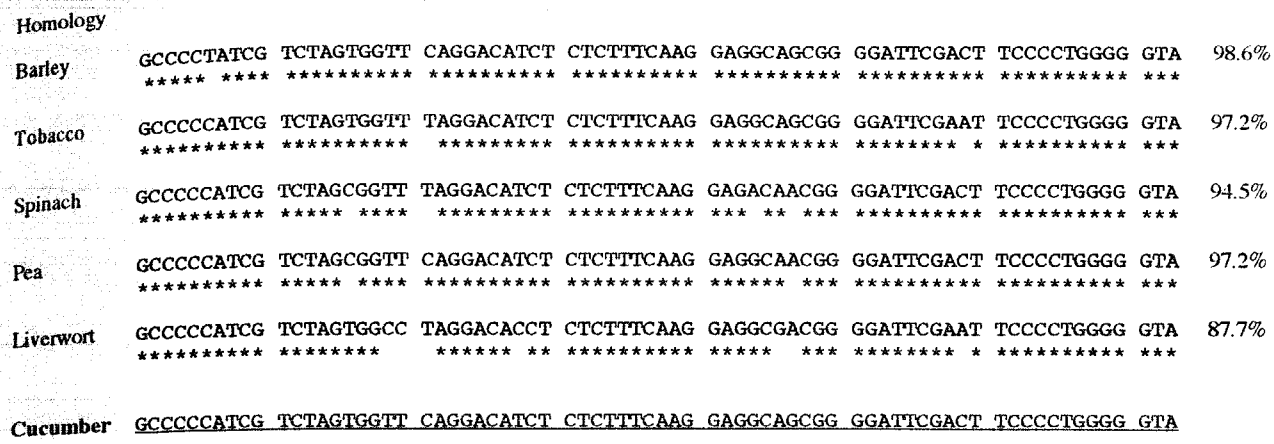


Figure II-8. Comparison of sequences of chloroplast $tRNA^{Glu}$ genes. The DNA sequence for cucumber chloroplast $tRNA^{Glu}$ was compared to those of barley, tobacco, spinach, pea and liverwort. The nucleotides identical to those of cucumber are marked by stars, and total homology is shown on the right side of the sequences.

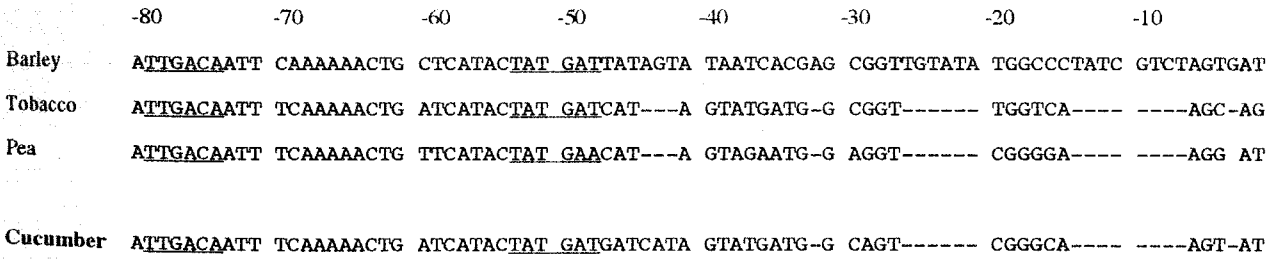


Figure II-9. Comparison of sequences immediately 5' to the $tRNA^{Glu}$ gene in the chloroplast. The region immediately 5' to the *trnE* cucumber chloroplast was compared to those of barley, tobacco and pea genes. Putative promoter sequences are underlined.

liverwort. The sequence immediately 5' to the *trnE* was highly homologous to those of all higher plants reported so far (Fig. II-9).

DISCUSSION

The study in this Chapter showed that a fraction bound to chlorophyllin-Sepharose column stimulated ALA synthesis *in vitro* and contained $tRNA^{Glu}$. This suggests that $tRNA^{Glu}$ participates in ALA synthesis in cucumber as reported for other plant species (Huang *et al.*, 1984; Weinstein *et al.*, 1986; Kannangara *et al.*, 1988). Genomic Southern hybridization showed that only one copy of $tRNA^{Glu}$ gene was encoded in the chloroplast genome. Thus, the product of this gene must be used for both ALA and protein biosynthesis.

The 1.3-kb fragment of chloroplast DNA contained two tRNA genes for glutamate (*trnE*, UUC) and threonine (*trnT*, GGU). In barley, *trnE* is closely linked to tyrosine and aspartate tRNA genes. $tRNA^{Glu}$, $tRNA^{Tyr}$ and $tRNA^{Asp}$ are processed from a single precursor RNA (Berry-Lowe, 1987). This is also the case with tobacco, spinach and pea (Holschuh *et al.*, 1984; Rasmussen *et al.*, 1984; Ohme *et al.*, 1985). In our experiments, *trnE* was located at the 5' end of the 1.3-kb fragment, and its upstream region could not be determined. However, a high sequence homology among plant species of the region immediately 3' to the *trnE* gene suggests the presence of a common regulatory mechanism for transcription. It might be considered that in cucumber chloroplast genome, $tRNA^{Tyr}$ and $tRNA^{Asp}$ genes are located at 5' upstream of *trnE*, and cotranscribed with *trnE* as a precursor RNA.

The *trnE* is 73 nucleotides long with the UUC anticodon expected for $tRNA^{Glu}$ species. The sequence of cucumber *trnE* is highly homologous to known chloroplast genes of other plant species. All of these tRNAs have an A:U base pair in position 53:61 which is replaced by G:C base pair in all tRNAs coding for other amino acids (Schön and Söll, 1988). This unusual A_{53}:U_{61} base

pair may be important for discrimination of this RNA by the barley glutamyl-tRNA reductase (Schön and Söll, 1988).

The expression of this gene by environmental factors has not clarified yet. In Chapter III, I described the examination of the effects of light and cytokinin on the level of $tRNA^{Glu}$ in plastids.

REFERENCE

Berry-Lowe, S. (1987) The chloroplast glutamate tRNA gene required for δ–aminolevulinate synthesis. *Carlsberg Res. Commun.*, **52**: 197-210.

Birnboin, H. C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.*, **7**: 1513-1523.

Blake, M. S., Johnston, K. H., Russel-Jones, G. J. and Gotschlich, E. C. (1984) A rapid sensitive method for detection of alkali phosphatase-conjugated anti-antibody on western blots. *Anal. Biochem.*, **136**: 175-179.

Dei, M. and Tsuji, H. (1987) Synthesis of 5-aminolevulinic acid by plastids isolated from cotyledons of greening cucumber seedlings pretreated with benzyladenine. *Plant Physiol. (Life Sci. Adv.)*, **6**: 63-68.

Grimm, B., Bull, A., Welinder, K. G., Gough, S. P. and Kannangara, C. G. (1989) Purification and partial amino acid sequence of the glutamate 1-semialdehyde aminotransferase of barley and synechococcus. *Carlsberg Res. Commun.*, **54**: 67-79.

Henikoff, S. (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene*, **28**: 351-359.

Holschuh, K., Bottomley, W. and Whitfield, P. R. (1984) Organization and nucleotide sequence of the genes for spinach chloroplast $tRNA^{Glu}$ and $tRNA^{Tyr}$. *Plant Mol. Biol.*, **2**: 313-317.

Huang, D. D., Wang, W. Y., Gough, S. P. and Kannangara, C. G. (1984) δ–Aminolevulinic acid-synthesizing enzymes need an RNA moiety for activity. *Science*, **255**: 1482-1484.

Kannangara, C. G. and Gough, S. P. (1977) Synthesis of δ–aminolevulinic acid and chlorophyll by isolated chloroplasts. *Carlsberg Res. Commun.*, **42**: 441-457.

Kannangara, C. G., Gough, S. P., Oliver, R. P. and Rasmussen, S. K. (1984) Biosynthesis of δ-aminolevulinate in greening barley leaves VI. activation of glutamate by ligation to RNA. *Carlsberg Res. Commun.*, **49**: 417-437.

Kannangara, C. G., Gough, S. P., Bruyant, P., Hoober, J. K., Kahn, A. and von Wettestein, D. (1988) $tRNA^{Glu}$ as a cofactor in δ-aminolevulinate biosynthesis: steps that regulate chlorophyll synthesis. *Trends Biochem. Sci.*, **13**: 139-143.

Laemmili, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.

Lew, R. and Tsuji, H. (1982) Effect of benzyladenine treatment duration on δ-aminolevulinic acid accumulation in the dark, chlorophyll lag phase abolition, and long term chlorophyll production in excised cotyledons of dark-grown cucumber seedlings. *Plant Physiol.*, **69**: 663-667.

Maniatis, T., Gritsch, E. F. and Sambrook, J. (1982) *Molecular cloning*. Cold Spring Harbor Laboratory Press, New York.

Mauzerall, D. and Granick, S. (1956) The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine. *J. Biol. Chem.*, **219**: 435-446.

Ohme, M., Kamogashira, T. and Sugiura, M. (1986) Structure and cotranscription of tobacco chloroplast genes for $tRNA^{glu}$ (UUC), $tRNA^{tyr}$ (GUA), and $tRNA^{asp}$ (GUC). *Nucleic Acids Res.*, **13**: 1045-1056.

Ohyama, K., Wetter, L. R., Yamano, Y., Fukuzawa, H. and Komano, T. (1982) A simple method for isolation of chloroplast DNA from *Marchantia polymorpha* L. cell suspension cultures. *Agric. Biol. Chem.*, **46**: 237-242.

Rasmussen, O. F., Stummann, B. M. and Henningsen, K. W. (1984) Nucleotide sequence of a 1.1 kb fragment of the pea chloroplast genome containing

three tRNA genes, one of which is located within an open reading frame of 91 codons., *Nucleic Acids Res.*, **12**: 9143-9152.

Schön, A. and Söll, D. (1988) Dual role of glutamyl-tRNA in barley chloroplast: involvement in chlorophyll and protein synthesis. *Arch. Biol. Med. Exp.*, **21**: 446-474

Urata, G. and Granick, S. (1963) Biosynthesis of α–aminoketones and the metabolisms of aminoacetone. *J. Biol. Chem.*, **238**: 811-820.

Wang, W. Y., Gough, S. P. and Kannangara, C. G. (1981) Biosynthesis of δ-aminolevulinate in greening barley leaves IV. isolation of three soluble enzymes required for the conversion of glutamate to δ–aminolevulinate. *Carlsberg Res. Commun.*, **46**: 243-257.

Weinstein, J. D., Mayer, S. M. and Beale, S. I. (1986) Stimulation of δ-aminolevulinic acid formation in algal extracts by heterologous RNA. *Plant Physiol.*, **82**: 1096-1101.

Chapter III

Regulation of ALA Synthesis by Light and Phytohormones

INTRODUCTION

The synthesis of ALA is known to be regulated by light. When etiolated leaves are exposed to the light, after the initial photoreduction of holochrome-associated protochlorophyllide, there is a lag phase of a few hours before the onset of rapid chlorophyll synthesis. The lag phase can be eliminated by preincubation of the tissues with exogenous ALA (Castelfranco *et al.*, 1974; Sisler and Klein, 1963). In the green algae, *Chlorella protothecoides* (Ochiai and Hase, 1970) and *Golenkinia minutissima* (Ellis *et al.*, 1975), exogenous ALA allows the cells to overcome the inhibition of chlorophyll synthesis caused by darkness and glucose or acetate. In *Cyanidium caldarium*, which normally requires light for chlorophyll and phycobilin synthesis, exogenous ALA in the light or dark causes the formation of large amounts of phycocyanobilin, the chromophore of phycocyanin, and some of the pigment is excreted into the medium (Troxler and Lester, 1967).

In tissues that normally require light for chlorophyll synthesis, the light is also required for continuous formation of ALA. When greening tissues are shifted to the dark, chlorophyll synthesis ceases immediately, but ALA synthesis ceases with a lag time (Fluhr *et al.*, 1975, Oh-hama and Senger, 1975). The period between the onset of darkness and the cessation of ALA formation may represent the time required for the buildup of a feedback of ALA synthesis. In *Euglena gracilis*, light induces both the tRNA and the enzymes required for ALA formation. The aminotransferase was the component most strongly affected (Mayer and Beale, 1990). A mutant of *Euglena* which lacks the

capacity for ALA synthesis by the C_5 pathway was shown to have all three enzymes for the C_5 pathway in the dark and lacked only the required $tRNA^{Glu}$. All three enzyme activities were induced by light (Huang and Castelfranco, 1990).

Beside the light, the synthesis of ALA is also regulated by phytohormones. Cytokinin, especially, is one of the most effective plant hormones for Chl synthesis. A variety of metabolic changes caused by application of cytokinins to plant tissues have been reported. Retardation of leaf senescence in intact plant is one of the numerous growth regulating effects of BA, one of cytokinins. It was suggested that this effect was associated with either a maintenance or an enhancement of chlorophyll synthesis (Fletcher, 1969, Adedipe *et al.*, 1971). It has also been observed (Stetler and Laetsch, 1965) that cytokinins are essential for chloroplast differentiation in tobacco tissue. In addition to these observations, it is reported that BA-induced stimulation of chlorophyll formation occurred in cucumber cotyledons (Sugiura, 1963, Fletcher and McCullagh, 1971). This stimulation may be due to increased synthesis of ALA because this is the rate limiting step in the biosynthesis of Chl.

The effect of light on the endogenous levels of cytokinins in seedlings was investigated by several analytical methods. With bioassays, light-induced increases in levels of cytokinins have been demonstrated in *Xanthium* leaves (van Staden and Wareing, 1972), mature poplar leaves (Hewett and Wareing, 1973), detached etiolated cotyledons of squash (Uheda and Kuraishi, 1977), *Amaranthus* seedlings (Kohler *et al.*, 1980), and cultured tobacco cells (Nishinari and Syono, 1980). New analytical methods which permit more accurate measurements of the levels of endogenous plant hormones confirmed a light-mediated increase in levels of cytokinins in *Sinapis alba* by enzyme-linked immunosorbent assay (Lejeune *et al.*, 1988) and in squash seedlings by gas chromatography-selected ion monitoring (Kuraishi *et al.*, 1991). Therefore,

cytokinins and light could act together in the formation of chlorophyll and functional plastids in etiolated plants (Uheda and Kuraishi, 1977, Longo *et al.*, 1981).

Although substantial works on the chloroplast biogenesis have been done with a number of monocot species, they are less sensitive to plant hormones than dicots in their response for the enhancement of the greening. A dicot cucumber is one of a species of Cucurbitaceae, and widely used to study the chloroplast development and chlorophyll biosynthesis, and it has been shown that after treatment of cucumber cotyledons with BA, the rate of ALA synthesis was doubled either in light or darkness (Fletcher *et al.*, 1973, Lew and Tsuji, 1982). Therefore, study on the ALA synthesizing system using cucumber is significant for the understanding of the regulation of ALA synthesis by light and hormones. However, there is little information about the ALA synthesizing system in dicots, especially on the involvement of $tRNA^{Glu}$ in ALA synthesis. As discussed in Chapter II, I determined the sequence of the gene for chloroplast $tRNA^{Glu}$ from cucumber chloroplasts.

In this Chapter, I described the effects of light and BA on ALA synthesizing system. The roles of both light and BA in modulating cellular levels of enzymes, their cofactors and substrates involved in the ALA synthesizing system are discussed.

MATERIALS AND METHODS

The followings were essentially the same as those described in Chapter II : Plant material and growth conditions, isolation of plastid and stroma proteins, and polyacrylamide gel electrophoresis and Western blot analysis

Chlorophyll measurement

Chlorophyll concentration was determined spectrophotometrically according to the method of Arnon (1949).

Assay for activity of ALA-synthesizing system *in vivo*

To examine the activity of ALA-synthesizing system, ALA accumulation was measured in the tissues treated with an inhibitor, LA, which inhibits ALA dehydratase. Cotyledons were excised without hypocotyl hook and placed abaxial side up on filter paper moistened with 1.6 ml of 0.1 M LA. They were incubated for 6 h under light illumination at 27°C, and then samples were collected and stored at -70°C. ALA accumulation was determined according to the procedure of Lew and Tsuji (1982). Plant tissues were homogenized in 4% TCA and centrifuged for 30 min at 10,000 x*g*. The resultant supernatant was collected and the volume was adjusted to 10 ml. The pH of the supernatant was adjusted to 4.6 by addition of 4.8 ml of 1 M Na-acetate, and then 0.3 ml of acetylacetone were added. The sample was heated to 100°C for 10 min. An equal volume of the sample and modified Ehrlich's reagent (Urata and Granick, 1963) were mixed, and absorbance at 553 nm was measured after 15 min.

GSA measurement

GSA was extracted and determined as follows. Cotyledons were homogenized in 10 ml of ice cold 0.1 N HCl containing 5% H_3PO_4, and centrifuged at 10,000 x*g* for 30 min. The supernatant was purified using a column of Dowex 50W-X8 (200-400 mesh), (0.7 × 2.0 cm), which had been washed with 3 ml of 1 N NaOH, then 3 ml of 0.2 M Na-citrate buffer (pH 3.1).

Ten ml of leaf extracts were loaded onto the column and washed with 8 ml of 0.2 M Na-citrate buffer (pH 3.1) followed by 1 ml of 0.2 M Na-citrate buffer (pH 4.1). The absorbed GSA was eluted with 3 ml of 0.2 M Na-citrate (pH 4.1).

For the determination of GSA concentration, a GSA solution was adjusted to pH 6.9 using 0.5 M Na_2HPO_4 and 0.5M NaH_2PO_4. Ethylacetoacetate (100 μl) was added and the mixture incubated for 20 min at 100°C. The solution was cooled to room temperature and an equal volume of modified Ehrlich's reagent was added. After 20 min, absorbance at 553 nm was measured against an appropriate control. The millimolar absorption coefficient of 1.0 was employed.

Assay for ALA synthesizing activity in plastid

ALA synthesizing activity in plastid was assayed by the method of Dei and Tsuji (1987). Incubation was done for 60 min at 29°C under the same light conditions as used for illumination of seedlings. Reaction medium contained 0.3 M glycerol, 0.1 M Tricine (pH 8.0), 1 mM EDTA, 25 mM $MgCl_2$, 1 mM L-glutamate, 5 mM ATP, 1.5 mM NADPH, 10 mM LA and isolated plastids (0.5-1.0 x 10^8 plastids equivalent to 2-4 mg protein). As Naito *et al.* (1979) reported that BA increased chloroplast number per cell, the plastids of the same number from BA-treated or untreated cotyledons were added to the reaction mixture. Reaction was terminated by adding 100 μl of 40% TCA. ALA was partially purified with a column of Dowex 50W-X8 (Duggan and Gassman, 1974, Wang *et al.*, 1981), and determined by measuring absorbance at 553 nm (A_{553}) of the eluate after reacting with Ehrlich-Hg reagent (Urata and Granick, 1963).

Assay for glutamyl-tRNA synthetase

Glutamyl-tRNA synthetase activity was determined by measuring [^{14}C]Glutamyl-tRNA formation from [^{14}C]L-glutamate. Stromal proteins (1 mg protein) were incubated for 30 min in 100 μl of reaction medium which contained 0.3 M glycerol, 0.1 M Tricine, pH 8.0, 1 mM EDTA, 25 mM $MgCl_2$,

[^{14}C]L-glutamate (92.5 kBq) and 5 mM ATP. An equal volume of phenol was added and the two phases were mixed thoroughly. After centrifugation, one-tenth volume of 3 M Na-acetate (pH 5.5) and 2.5 volumes of ethanol were added to the aqueous fraction, and was kept at -70°C for 1 h. The precipitated RNAs were collected by centrifugation, dried in a vacuum desiccator. The precipitate finally obtained was dissolved in 50 μl of water, and 5 ml of scintillation cocktail was added. The radioactivity was measured by a liquid scintillation counter.

Assay for glutamyl-tRNA reductase

Since spectroscopic detection of Ehrlich reagent-reacting ALA-pyrrole is much more sensitive than that of GSA-pyrrole, glutamyl-tRNA reductase activity was determined by measuring the ability of stromal proteins to form ALA in the presence of sufficient amounts of both glutamyl-tRNA and GSA aminotransferase. To keep glutamyl-tRNA reduction step to be rate-limiting one, sufficient amount (100 μg) of GSA aminotransferase from *Synechococcus* PCC 6301 (Grimm *et al.*, 1991) was supplemented. Glutamyl-tRNA was prepared according to the procedure to assay of glutamyl-tRNA synthetase with 1 mM L-glutamate as a substrate. Incubation was carried out at 28°C for 30 min in 300 μl of reaction mixture containing 0.3 M glycerol, 0.1 M Tricine, pH 8.0, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM NADPH, 1 A_{260} unit of glutamyl-tRNA, 5 mM of LA, 100 μg of GSA aminotransferase and stromal proteins (1 mg protein). ALA extraction and determination were performed according to the procedure of assay for ALA synthesizing activity which is described in Chapter II.

Assay for GSA aminotransferase

Aminotransferase activity was assayed by measuring conversion of chemically synthesized GSA (Gough *et al.* 1989) to ALA by stromal proteins. Incubation was carried out at 28°C for 30 min in 200 μl of reaction mixture containing 0.3 M glycerol, 0.1 M Tricine, pH 8.0, 1 mM EDTA, 1 mM $MgCl_2$, 5

mM of LA, 300 μM GSA and stromal proteins (1 mg protein). ALA extraction and determination were performed as described above. Because non-enzymatically conversion of GSA to ALA was observed, GSA aminotransferase activity was determined by calculating the net ALA formation by subtracting non-enzymatic formed ALA from total ALA.

Determination of glutamate in stroma

Determination of glutamate was carried out by two methods. One is the enzymatic determination with glutamate dehydrogenase, diaphorase and tetrazolium salts (Beutler and Michal, 1974) and the other is HPLC analysis of fluorescent derivatives of amino acids (Watanabe and Imai, 1984).

(1) Determination by enzymatic method

Glutamate was extracted from 100 μl of plastid suspension with an equal volume of 1 M perchloric acid. The mixture was centrifuged at 3,000 x*g* for 10 min. The pH of the resulting supernatant was adjusted to 9.0 with 40 μl of 1.93 M K_3PO_4. After 10 min, the mixture was recentrifuged, and resulting supernatant was used for assay.

The assay mixture contained 57 mM triethanolamine hydrochloride, 14 mM phosphate, pH 8.6, 0.38 mM NAD, 0.068 mM iodonitrotetrazolium chloride, 0.14 U/ml diaphorase, and extracted supernatant (up to 50 μM glutamate). After mixing, the absorbance at 492 nm was measured. Reaction was initiated by the addition of 14 U/ml glutamate dehydrogenase, and change in absorbance was read several times at intervals of 3 min. Standard glutamate solution (20 μg/ml) was used for calibration.

(2) HPLC analysis of fluorescent derivative of glutamate

To rupture plastids, suspension of plastids was treated with a glass homogenizer. The homogenate was centrifuged at 12,000 x*g* for 30 min. The resultant supernatant (stromal fraction) was used for analysis. Amino acids were

extracted from the stromal fraction with ethanol and reacted with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) at 60°C and at pH 8.0 for 1 min. The fluorescent derivatives were separated by HPLC on a Richrospher 100 RP-18 column, and emission at 530 nm (excitation at 470 nm) was detected by a fluorescence monitor (Shimadzu RF-550, Kyoto). Derivative of authentic glutamate was subjected to HPLC. The glutamate derivative (NBD-Glu) eluted at about 8.4 min. The ratio of peak area of NBD-Glu to that of the internal standard (ε-aminocaproic acid derivative) was calculated for each concentration of glutamate used. The ratios were plotted against the concentration of glutamate, a linear relationship being observed in the range of 0.1-20 pmol.

Determination of ATP in stroma

ATP was extracted from plastids as described by Inoue *et al.* (1978). Two hundred μl of plastid suspension were mixed with 1.3 ml of 10 mM TES, pH 7.4, and 40 mM $MgSO_4$. The mixture was heated at 100°C for 5 min, and then cooled on ice. After centrifugation, 100 μl of supernatant were used for assay. ATP was quantitated with a luciferin-luciferase assay kit (Lucifer-KP; Kikkoman, Chiba), and a fluorescence spectrophotometer, (Model 850, Hitachi Co., Tokyo) equipped with a photon counting unit. Equal volume of the supernatant and luciferin-luciferase solution were mixed. ATP concentration was determined by counting luminescent intensity during 20 to 40 s after initiation of the reaction. Authentic ATP solution was used as standard.

Determination of NADPH in stroma

NADPH was extracted from plastids with alkali under various conditions, with concomitant decomposition of the oxidized forms of NAD and NADP. Half ml of 1 N KOH in ethanol was added to 1 ml of plastids suspension. The mixture was allowed to stand for 30 min at room temperature, and then cooled on ice. The pH of the mixture was adjusted to 7.8 by the addition of 1.5 ml of

0.5 M triethanolamine, 0.4 M KH_2PO_4 and 0.1 M K_2HPO_4. After 10 min, denatured protein was removed by centrifugation at 20,000 x*g* for 20 min. The resulting supernatant was used for the measurements.

Measurement of NADPH was done by enzymatic cycling according to the method of Passonneau and Lowry (1974). Cycling reagent containing 81 mM Tris-HCl (pH 8.0), 0.8 mM glucose-6-phosphate, 40 mM ammonium acetate, 80 μM ADP, 4 mM 2-oxoglutarate, 7 U/ml glucose-6-phosphate dehydrogenase, and 9 U/ml glutamate dehydrogenase. To 0.1 ml of cycling reagent, 20 μl of the sample were added. The mixture was incubated at 38°C for 60 min and placed in boiling water bath for 2 min, followed by the addition of 1 ml of 6-phosphogluconate reagent (18 mM Tris-HCl, pH 8.0, 27 mM ammonium acetate, 90 μM EDTA, 18 μM NADP, 0.018% BSA, and 6 mU/ml 6-phosphogluconate dehydrogenase). The mixture was incubated for 30 min at room temperature. After reaction, fluorescence intensity (excitation at 360 nm and emission at 460 nm) was measured with the fluorescence spectrophotometer, (Model 850, Hitachi Co., Tokyo). Authentic NADPH and 6-phosphogluconate was used as standard.

Determination of Mg in stroma

Plastids were isolated as described above in the buffer system without $MgCl_2$. Stromal fraction was obtained by alkaline extraction to avoid a release of Mg into stroma by dechelation of chlorophyll. Plastids (100 μl suspension) were ruptured by addition of 50 μl 1 M KOH, and centrifuged at 10,000 x*g* for 10 min. Resulting supernatant was diluted 100-fold with ultra pure water. The level of Mg was determined in inductivity coupled plasma atomic emission spectroscopy, (Model SPS 1500 VR, Seiko Co,,). Authentic Mg standard solutions (Wako Co.) were used for calibration.

Preparation of total RNAs

Total RNAs were prepared according to Iwasaki *et al.* (1990) with modifications. About 0.5 g of cotyledons was homogenized with a Polytron homogenizer in 7 ml of homogenizing medium containing 3.5 ml buffer (4 M guanidine thiocyanate, 20 mM Tris-HCl, pH 8.0, 2% sarcosyl and 2% 2-mercaptoethanol) and 3.5 ml phenol:chloroform (1:1, v/v). The homogenate was centrifuged at 2,500 x*g* for 10 min at 4°C. The aqueous phase was mixed with an equal volume of phenol-chloroform. Extraction was repeated twice more, and the aqueous fraction was then extracted with chloroform: isoamyl alcohol (24:1, v/v). Total nucleic acids in the aqueous phase were precipitated with ethanol. After dissolving the ethanol precipitate in water, total RNAs were used for Northern blot analysis.

When total RNAs were prepared from plastids, RNAs were extracted from isolated plastids with an equal volume of phenol which had been saturated with 10 mM Tris (pH 8.0) and 1 mM EDTA. Phenol was removed from the aqueous phase by washing twice with chloroform:isoamylalcohol (24:1, v/v). One-tenth volume of 3 M Na-acetate (pH 5.5) and 2.5 volumes of ethanol were added to the aqueous fraction, which was then kept at -70°C for 1 h. The precipitated RNAs were collected by centrifugation, dried in a vacuum desiccator, and dissolved in distilled water. The recovery of RNA from plastids was more than 90%.

Northern blot analysis

The level of mRNA of glutamyl-tRNA reductase was analyzed by Northern blotting according to the procedure of Maniatis *et al.* (1982). The obtained RNA solution was further purified by precipitation with 2.5 M LiCl. After dissolving the precipitate, 20 μg of total RNAs were denatured and subjected to 1% formaldehyde-agarose gel. RNA was transferred to a nylon membrane by capillary reaction, and the blots were screened with the ^{32}P-

labelled cDNA fragment of cucumber *hemA*. The membrane was hybridized for 18 h at 65°C in blotting buffer, and was washed in 2xSSC containing 0.1% SDS two times for 15 min at 65°C and in 0.2 × SSC containing 0.1% SDS two times for 30 min each at 40°C.

For the determination of the levels of tRNAs, cucumber total RNAs (10 μg) were separated by electrophoresis on 4% NuSieve agarose (FMC Bioproducts) and transferred to a nylon membrane by capillary reaction as described by Maniatis *et al.* (1982). The conditions of hybridizations were the same as described in Southern blot analysis in Chapter II and hybridizations were detected by autoradiography.

RESULTS

1. Effects of BA and light on Chl and ALA synthesis

Effects of BA on the levels of Chl and ALA in greening cotyledons

Since no accumulation of Chl and its precursor has been known in the etiolated seedlings, I re-examined the stimulatory effect of BA on the accumulation of Chl and ALA during continuous illumination. The effect of BA on Chl and ALA synthesis in the light was dependent on the dark preincubation with BA (data not shown), and was in accordance with results from Fletcher and McCullagh (1971). Therefore, cucumber cotyledons were excised and preincubated with 50 μM BA for 12 h in the dark, and then illuminated. Fig. III-1 shows the effect of BA on Chl accumulation during greening of cotyledons excised from 4-day-old etiolated seedlings. The stimulation of Chl accumulation and the abolition of the lag for Chl formation were observed with BA-treated samples as reported previously (Lew and Tsuji, 1982). BA also stimulated ALA accumulation, in accordance with previous observations (Fletcher and McCullagh, 1971, Lew and Tsuji, 1982). After 6 h of illumination, 212.0 nmol ALA per g fresh weight accumulated in BA-treated cotyledons, while 124.2 nmol ALA per fresh weight in the control.

BA and light stimulate ALA synthesis

To test whether the stimulation of ALA accumulation by BA is due to the acceleration of synthesis of ALA or not, the rate of synthesis of ALA in BA-treated or untreated cotyledons in the dark and light was examined. Since almost no accumulation of ALA was observed in the cotyledons in the dark as described above, a red light pulse was irradiated to induce ALA accumulation during the subsequent dark period. Cotyledons were excised and incubated in the darkness up to 12 h. The competitive inhibitor for ALA dehydratase, LA,

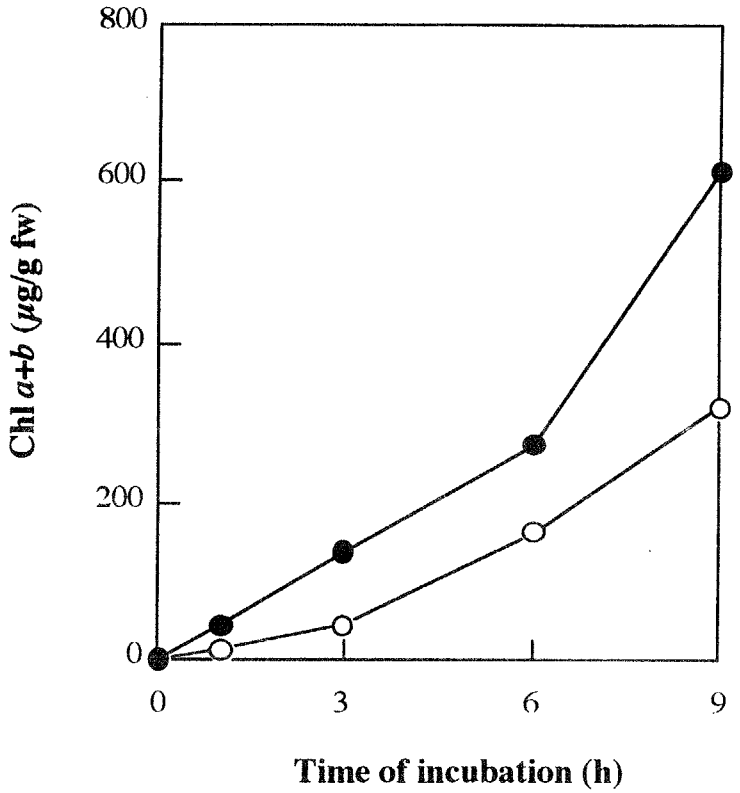


Figure III-1. Effect of BA on Chl accumulation in excised cotyledons during 9 h of continuous illumination. After excision, the cotyledons were incubated with H_2O or BA for 12 h in the dark. Each point represents the means of duplicate samples. ● , BA; ○ , control.

was added to cotyledons 2 h prior to harvest cotyledons to cause ALA accumulation. Almost no accumulation of ALA occurred in the dark with or without BA treatment (data not shown), but ALA accumulation was found to occur after a red light pulse was irradiated during the subsequent dark period (data not shown, cf. Klein *et al.*, 1975). A red light pulse was irradiated after 1 h of LA treatment. Fig. III-2 shows the effect of BA on ALA synthesizing activity during dark incubation. Maximum stimulation of ALA synthesizing activity occurred after 6 to 9 h of the dark incubation (Fig. III-2). BA enhanced ALA synthesis, and a significantly greater amount of ALA was produced compared with controls. This result indicates that the stimulation of ALA synthesis by BA is induced during dark incubation.

In experiment in the light (Fig. III-3), cotyledons were incubated with or without BA in the dark for 12 h, and then transferred to the light. In BA-treated tissues, the substantial ALA synthesis was observed after 1 h of light illumination. The rate of synthesis of ALA after 1 h of illumination was about 2-fold of untreated controls, and this may contribute to the BA-induced abolition of lag phase for chlorophyll synthesis. The maximum rate of ALA synthesis occurred after 3 h of illumination, and then the rate of ALA synthesis gradually decreased. The rate of ALA synthesis after 3 h was about 2-fold of untreated controls. In untreated tissue, the rate of synthesis of ALA also increased after light illumination. The activity reached maximum after 3 h of illumination, and then gradually decreased. The change of the rate of ALA synthesis in both BA-treated and untreated tissues was in a manner similar to Chl synthesis activity (data not shown), indicating that the levels of ALA formation determine the Chl production.

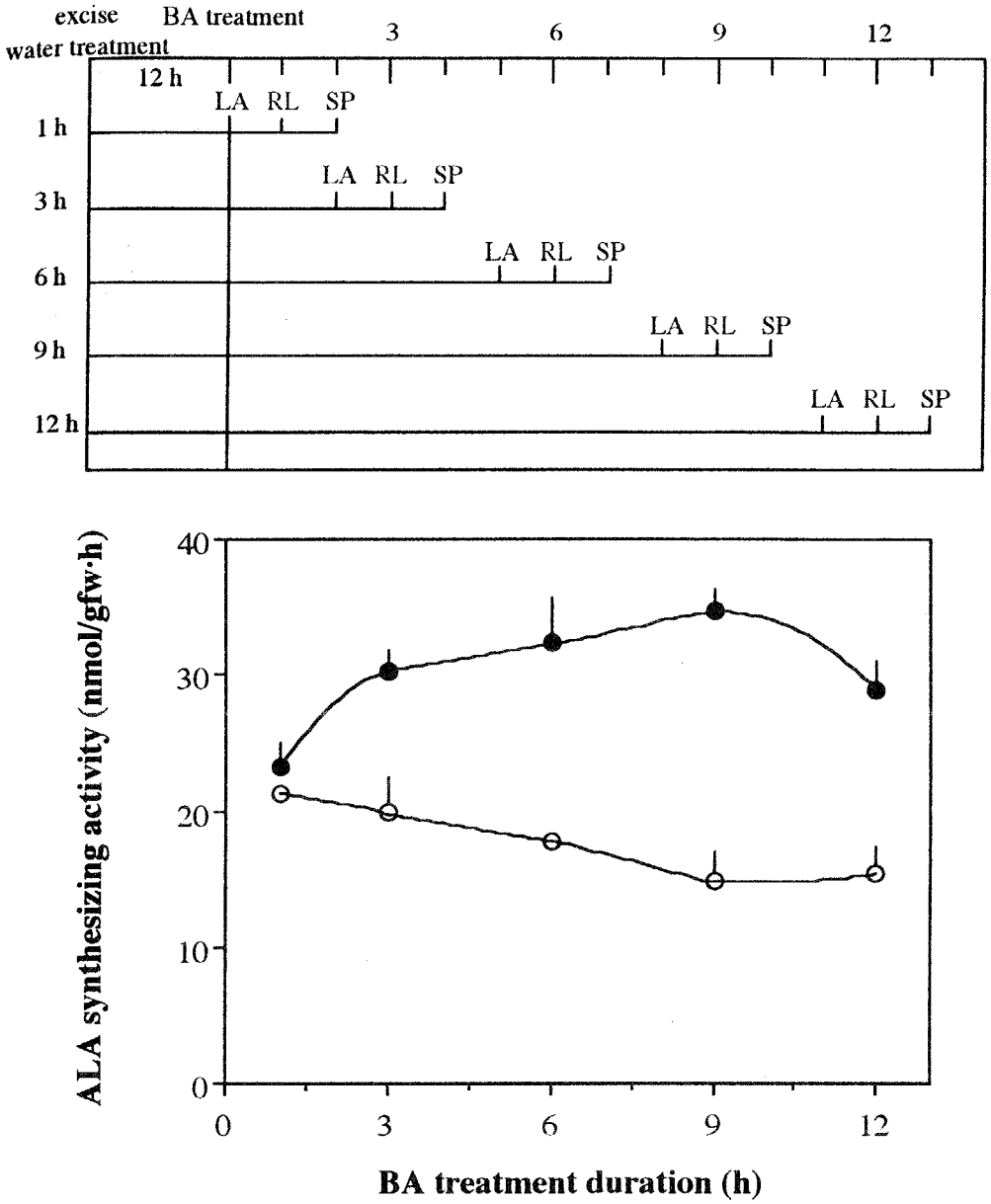


Figure III-2. Effects of BA on ALA synthesis during dark incubation. Upper panel, regime of treatment. After excision, the cotyledons were incubated with H_2O for 12 h. Then, cotyledons were incubated with H_2O or 50 μM BA for the indicated durations of treatment up to the onset of the red-light pulse (RL). LA was added 1 h prior to the red-light pulse (LA), and the samples were harvested 1 h after the red-light pulse (SP). Lower panel, ALA synthesizing activity. H_2O- (○) and BA- (●) treated cotyledons. Data represent means and range for two replicate experiments.

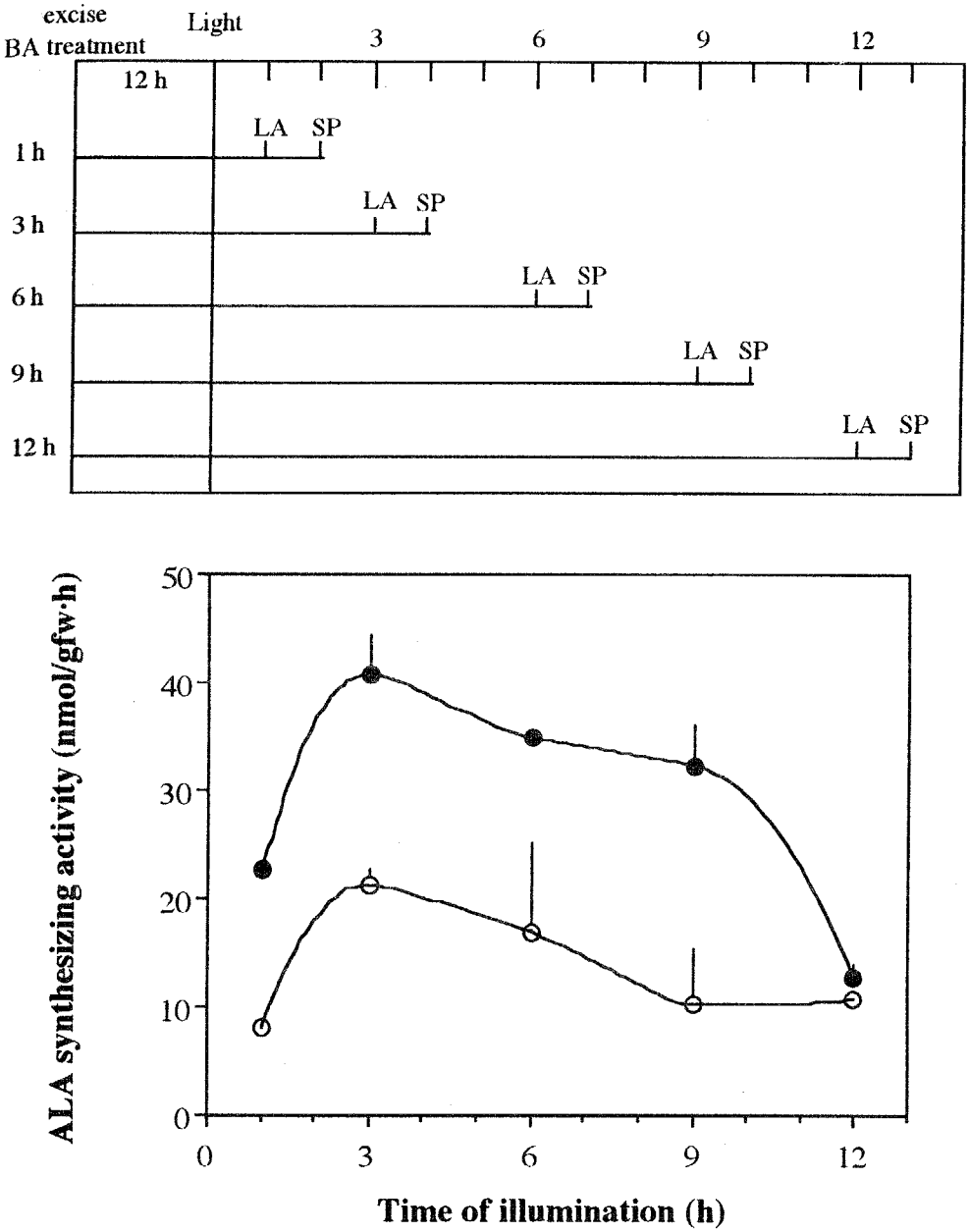


Figure III-3. Effects of BA on ALA synthesis during light incubation. Upper panel, Regime of treatments. After excision, the cotyledons were incubated with H_2O or BA for 12 h in darkness. Then cotyledons were exposed continuous light illumination and were incubated shown up to the time of the LA addition (LA). The samples were harvested 1 h after the LA addition (SP). Lower panel, ALA synthesizing activity. H_2O- (o) and BA- (●) treated cotyledons. Data represent means and range for two replicate experiments.

Effects of inhibitors of protein synthesis on ALA formation

The effects of inhibitors of protein synthesis on BA- and light-induced stimulation of ALA accumulation were analyzed. Cycloheximide and chloramphenicol were used as cytoplasmic and plastidic protein synthesis inhibitors, respectively. Excised cotyledons were treated with each inhibitor and BA and incubated for 12 h in the dark, and then illuminated. After 5 h of dark incubation, and at 1 h of light illumination, the cotyledons were treated with LA (in case of dark incubation, a red light treatment was followed), and ALA accumulation was assayed as described before.

When cotyledons were treated with 2 μg/ml cycloheximide, the BA-induced stimulation of ALA accumulation was abolished to the untreated levels in the dark (Fig. III-4A) and light (Fig. III-4B). By the treatment with 10 μg/ml cycloheximide, ALA accumulation was almost completely inhibited with or without BA treatment, and in the dark and light. These results indicate that the stimulation of ALA synthesis requires cytoplasmic protein synthesis. In contrast, almost no inhibition of ALA accumulation was observed by the treatment with 20 μg/ml of chloramphenicol with or without BA in the dark and light (Fig. III-4A, B). By the treatment with 100 μg/ml of chloramphenicol, ALA accumulation was substantially inhibited with or without BA-treatment. In particular, in cotyledons incubated in the light, the inhibition of ALA accumulation seemed more drastic than that in the dark. These results indicate that the stimulation of ALA accumulation by light and BA more depend upon the cytoplasmic protein synthesis than that of plastid and are in good agreement with the suggestion that the three enzymes involved in ALA synthesis (glutamyl-tRNA synthetase, glutamyl-tRNA reductase and GSA aminotransferase) are encoded by nuclear DNA (Jahn *et al.*, 1992). However, since chloramphenicol inhibited stimulation of ALA accumulation in the light

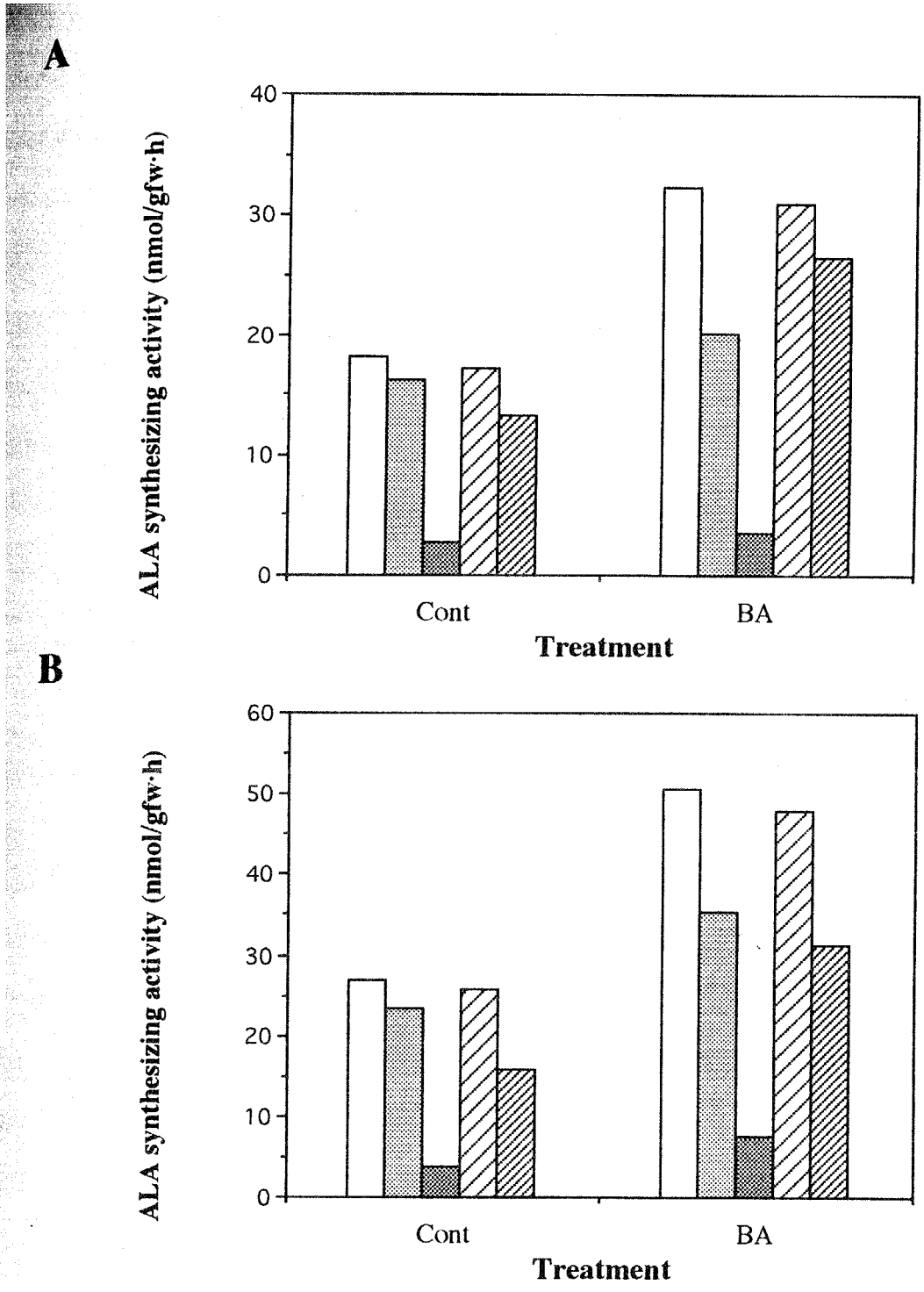


Figure III-4. Effects of inhibitors of protein synthesis on ALA synthesizing activity. Cotyledons were excised and incubated with 50 μM BA or H_2O. Cycloheximide, 2 μg/ml (▩) and 10 μg/ml (▩); chloramphenicol, 20 μg/ml (▨) and 100 μg/ml (▨); or H_2O (□). A, Cotyledons were preincubated with water for 12 h and then incubated with BA or H_2O for 6 h in the dark. After 5 h of dark incubation, cotyledons were treated with LA, followed by red-light pulse as described in Fig. III-2. B, Cotyledons were incubated with BA or H_2O for 12 h in the dark and then illuminated for 2h. At 1h of light illumination, LA was added. The results are the mean of duplicate experiments.

more drastically, the plastid development could also be related to the stimulation of ALA synthesis.

2. ALA synthesis in greening plastids

ALA synthesis in greening plastids

To elucidate the effects of BA and light on ALA synthesis more in detail, I examined the activity of ALA-synthesizing system in isolated plastids. Intact plastids were isolated from greening cucumber, and activities of ALA synthesis were investigated in terms of overall activity of ALA synthesis and activities of three enzymes involved in the ALA-synthesizing system. Table III-1 shows the rate of synthesis of ALA in the light in intact plastids isolated from greening cucumber cotyledons that had been treated with BA and in plastids from untreated controls. ALA synthesizing activity was increased by light illumination.

BA-treated samples showed about 2.7-fold the ALA-synthesis activity of untreated controls at all greening stages. ALA synthesizing activity in BA-treated tissues was also increased by light illumination.

Effects of BA and light on activities of the three enzymes catalyzing ALA synthesis in cucumber

The effects of BA and light on the activities of three enzymes catalyzing ALA synthesis (*i.e.*, reactions catalyzed by glutamyl-tRNA synthetase, glutamyl-tRNA reductase and GSA aminotransferase) were examined with stromal fractions prepared from etiolated and 8 h illuminated cotyledons treated with or without BA. Firstly, glutamyl-tRNA synthetase activity was assayed by measuring the incorporation of radioactivity of [^{14}C]L-glutamate into the nucleic acid fraction of plastids. The incorporation of radioactivity was observed when the reaction mixtures were incubated with ATP (data not shown). Both stromal fractions from etiolated and greening plastids from BA-treated tissues showed the activities about 2-fold of untreated controls (Table

Table III-1

ALA synthesizing activity in plastids from greening cucumber cotyledons that had been treated with BA for 12 h.

Treatment	ALA synthesizing activity (nmol/10^9 plastids·h)		
	Time of illumination (h)		
	0	1.5	8
Control	2.86	4.17	4.70
BA	4.76	8.27	10.23

The values are the means of results from three replications.

III-2). Light substantially increased the activity of glutamyl-tRNA synthetase in BA-treated and untreated fractions. In this assay, the endogenous $tRNA^{Glu}$ was utilized as substrate of glutamyl-tRNA synthetase. To determined the net activity of glutamyl-tRNA synthetase, the endogenous RNAs were digested by the treatment with RNase A. The stromal fractions were then treated with RNase inhibitor, and the same amount of the total RNAs prepared from untreated greening plastids were added and incubated under the same condition. The RNase A-treated fractions had almost no activity of glutamyl-tRNA synthetase (Table III-2). When equal amount of the total RNAs was added to the reaction mixtures, every fraction showed almost the equal activity of glutamyl-tRNA synthetase. This indicated that the BA- and light-induced stimulation of activity of glutamyl-tRNA synthetase is due to an increase of endogenous level of $tRNA^{Glu}$, but not the enzyme itself.

Secondly, the effects of BA and light on the activity of glutamyl-tRNA reductase were examined. As shown in Table III-3, the activity of reductase was increased with light illumination. BA-treated samples showed 2.2- and 1.8-fold the reductase activity of untreated controls of dark and light illuminated samples, respectively.

Thirdly, the activity of GSA aminotransferase was assayed as shown in Table III-4. The activity of GSA aminotransferase was not changed by both light illumination and BA treatment. This result suggested that the site of action of light and BA on the ALA synthesizing system is located before the step of GSA aminotransferase.

3. Effects of BA and light on the expression of the enzymes involved in ALA biosynthesis

Effect of BA on the GSA accumulation in greening cotyledons

To examine the stimulation site of BA, I tested the effect of gabaculine,

Table III-2

Effects of BA and light on the activty of glutamyl-tRNA synthetase in stromal fractions from plastids from greening cucumber cotyledons that had been treated with BA for 12 h.

Treatment	Glutamyl-tRNA formed (cpm/mg protein·h)	
	Time of illumination (h)	
	0	8
Control		
None[a]	4,578	7,875
+RNase A[b]	NA[d]	1,756
+total RNA[c]	8,673	9,732
BA		
None	10,540	14,497
+RNase A	NA	1,404
+total RNA	9,020	9,822

a, Stormal fractions were incubated as described in Materials and Methods.

b, Stromal fractions were incubated with 1μg/ml RNase A for 30 min at 28°C, followed by the treatment with 10 U of RNase inhibitor for 15 min.

c, Ten μg of total RNA from greening plastids were added to the RNase- and RNase inhibitor-treated fractions.

d, Not assayed.

Table III-3

Effects of BA and light on the activity of glutamyl-tRNA reductase in stromal fractions from plastids from greening cucumber cotyledons that had been treated with BA for 12 h.

Treatment	Glutamyl-tRNA reductase activity (nmol ALA/mg protein·h)	
	Time of illumination (h)	
	0	8
Control	0.077	0.214
BA	0.171	0.391

The values are the means of results from duplicates samples.

Table III-4

Effects of BA and light on the activity of GSA aminotransferase in stromal fractions from plastids from greening cucumber cotyledons that had been treated with BA for 12 h.

Treatment	GSA aminotransferase activity (nmol ALA/mg protein·h)	
	Time of illumination (h)	
	0	8
Control	3.28	2.73
BA	2.93	3.11

The values are the means of results from duplicates samples.

an inhibitor of Chl biosynthesis in higher plants and algae. The results shown in Fig. III-5 confirmed the inhibition of the greening of etiolated cotyledons.

It has been reported that the site of inhibition of gabaculine is GSA aminotransferase, and GSA accumulated in gabaculine-treated tissues (Kannangara and Schouboe, 1985). In the gabaculine-treated cotyledons, significant amount of GSA accumulated under the exposure of light (Fig. III-6), while no GSA accumulation was observed in the dark (data not shown). The GSA content increased with the time of illumination and reached steady state after 6 h of light. BA treatment stimulated the GSA accumulation in gabaculine-treated cotyledons in the light. And at 6 h of light, BA more than doubled the amount of GSA formed. This shows that the stimulation of ALA synthesis by BA occur before the step of aminotransferase, and is in accordance with the results from the assay of GSA aminotransferase (Table III-4).

No effects of light and BA on the levels of glutamyl-tRNA synthetase and GSA aminotransferase

The effects of light and BA on the levels of enzymes involved in ALA synthesis were analyzed by Western blotting with antibodies against glutamyl-tRNA synthetase and GSA aminotransferase.

The levels of glutamyl-tRNA synthetase were not augmented by light illumination (Fig. III-7). BA did not affect the levels of this enzyme. This result supports the previous indication that glutamyl-tRNA synthetase itself was not responsible for the action of light and BA (Table III-2). Similarly the levels of GSA aminotransferase were not changed by both light illumination and BA treatment (Fig. III-8). In higher plants, GSA aminotransferase does not require any cofactor for their activity. Together with the previous results (Table III-4, Fig. III-6), this result confirms the previous assumption that the site of action of light and BA on ALA synthesizing system is located before the step of GSA aminotransferase.

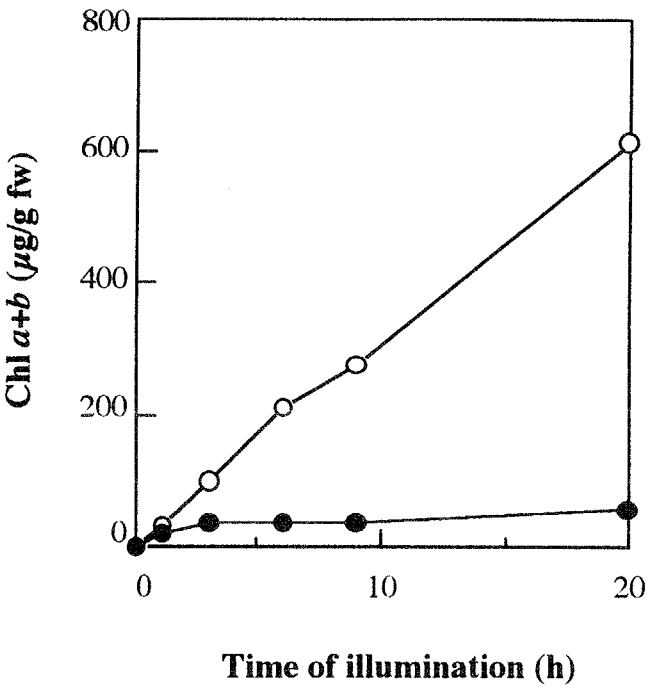


Figure III-5. Inhibition of Chl synthesis by gabaculine in excised cotyledons. 500 μM gabaculine was added at the beginning of illumination. ●, Gabaculine; ○, control.

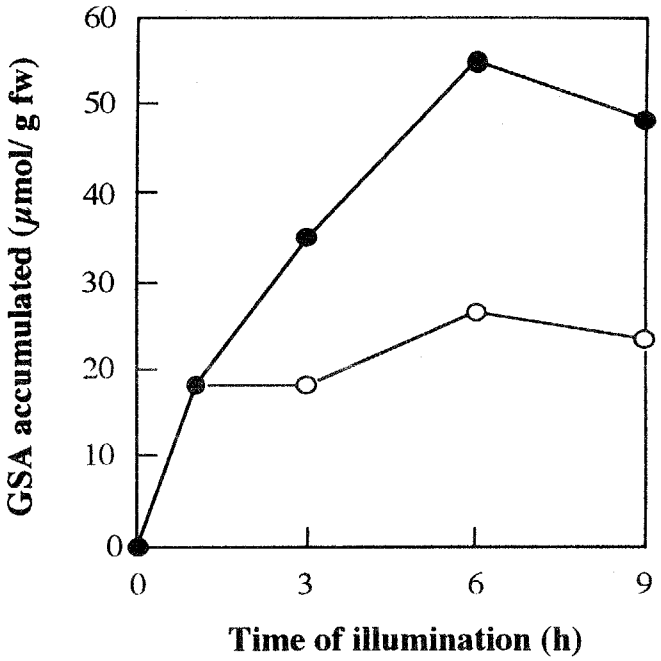


Figure III-6. Effect of BA on GSA accumulation in excised cotyledons in the presence of gabaculine during 9 h of illumination. ●, BA; ○, control.

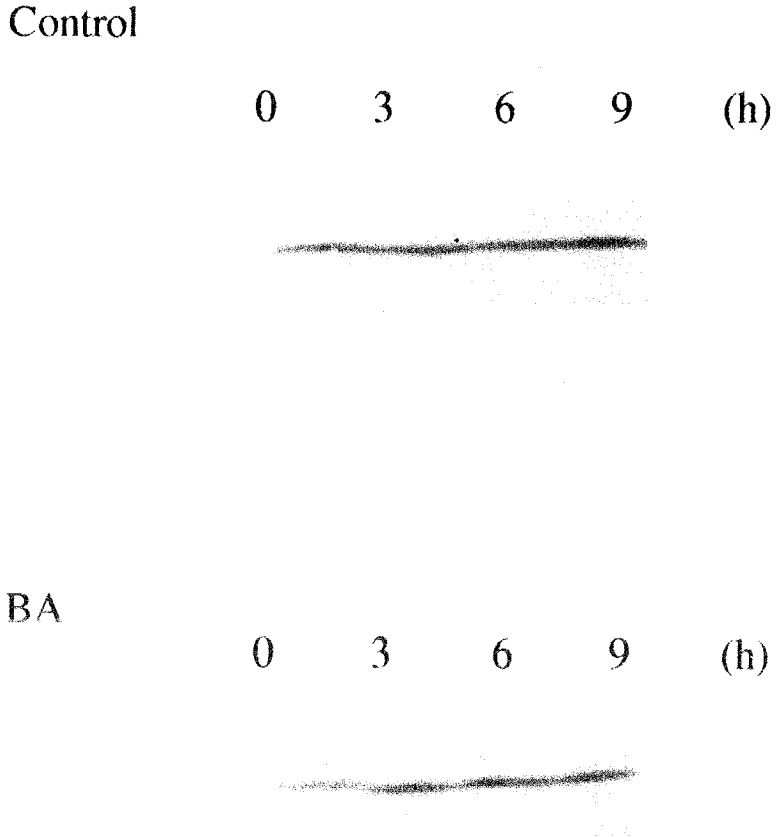


Figure III-7. Levels of glutamyl-tRNA synthetase from BA- (lower) and from water-treated cotyledons (upper). Cotyledons were sprayed with a 50-μM BA solution or with water, incubated for 12 h in darkness, and then illuminated for the indicated time. Total proteins were extracted from cotyledons, separated by SDS-PAGE, and electroblotted onto nitrocellulose membranes. Ten μg of the total proteins were loaded on gels. Blots were probed with antibody against glutamyl-tRNA synthetase. Immune complexes were visualized by use of a second antibody conjugated with alkaline phosphatase.

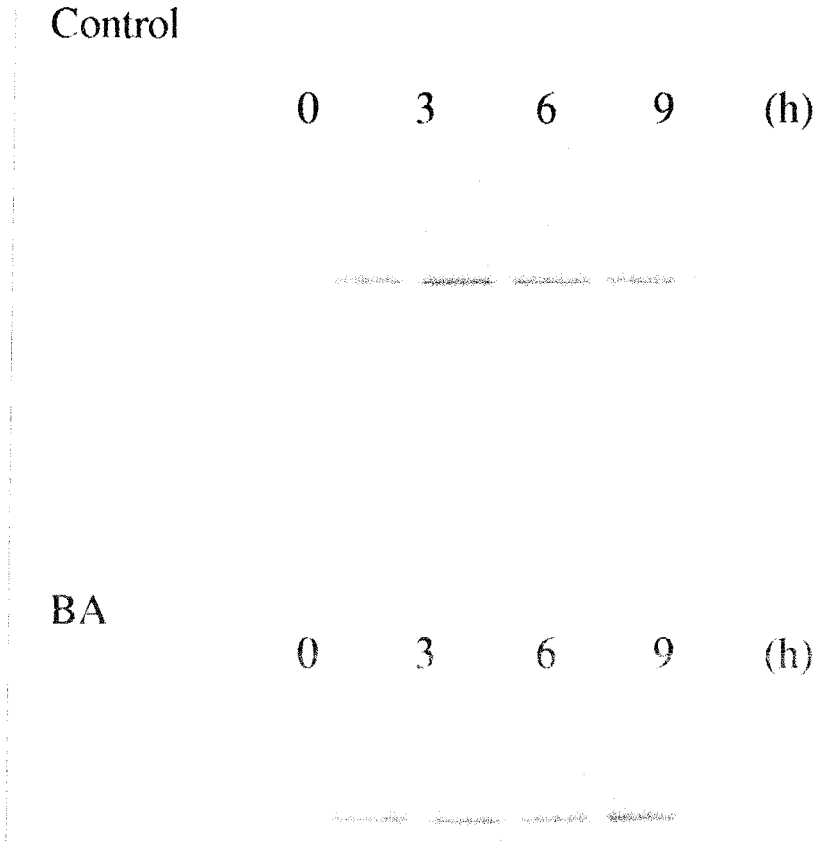


Figure III-8. Levels of GSA aminotransferase from BA- (lower) and water-treated cotyledons (upper). Cotyledons were sprayed with a 50-μM BA solution or with water, incubated for 12 h in darkness, and then illuminated for the indicated time. Total proteins were extracted from cotyledons, separated by SDS-PAGE, and electroblotted onto nitrocellulose membranes. Ten μg of the total proteins were loaded on gels. Blots were probed with antibody against GSA aminotransferase. Immune complexes were visualized by use of a second antibody conjugated with alkaline phosphatase

Northern blot analysis of glutamyl-tRNA reductase gene

The gene encoding glutamyl-tRNA reductase is named *hemA*. The *hemA* gene was isolated from *Arabidopsis thaliana* and cucumber by the complementation of the *hemA* mutation from *E. coli*. By using *hemA* gene from cucumber as a probe, the effects of light and BA on the expression of glutamyl-tRNA reductase gene were investigated by Northern blot analysis. BA increased the levels of mRNA of glutamyl-tRNA reductase in the dark, while the expression of this mRNA was remained at low levels in untreated controls (Fig. III-9A). When cotyledons were exposed to the light, the significant increase of glutamyl-tRNA reductase mRNA was observed (Fig. III-9B). The levels of the mRNA increased with illumination, and maintained the same levels during 3 to 12 h of illumination. After 24 h, the levels of glutamyl-tRNA reductase mRNA slightly decreased.

BA also increased the level of glutamyl-tRNA reductase mRNA under light illumination. The extent of BA-induced increase in the light was not so drastic as that in the dark. As shown in Fig. III-9A, the levels of glutamyl-tRNA reductase mRNA had already increased by the preincubation with BA in the dark (Fig. III-9B). Upon illumination, the level of this mRNA increased. The pattern of expression in BA-treated tissue was similar to that of water control and seemed to match with the rate of synthesis of ALA in BA-treated samples (cf. Fig. III-3). These results suggested that light and BA regulate the expression of glutamyl-tRNA reductase. In fact, light and BA increased the activity of glutamyl-tRNA reductase (Table III-3).

Northern blot analysis of $tRNA^{Glu}$

Changes in the expression levels of $tRNA^{Glu}$ by light and BA treatment were examined. Total RNAs were isolated from etiolated cucumber cotyledons illuminated for various periods for 12 h. A single band was detected by

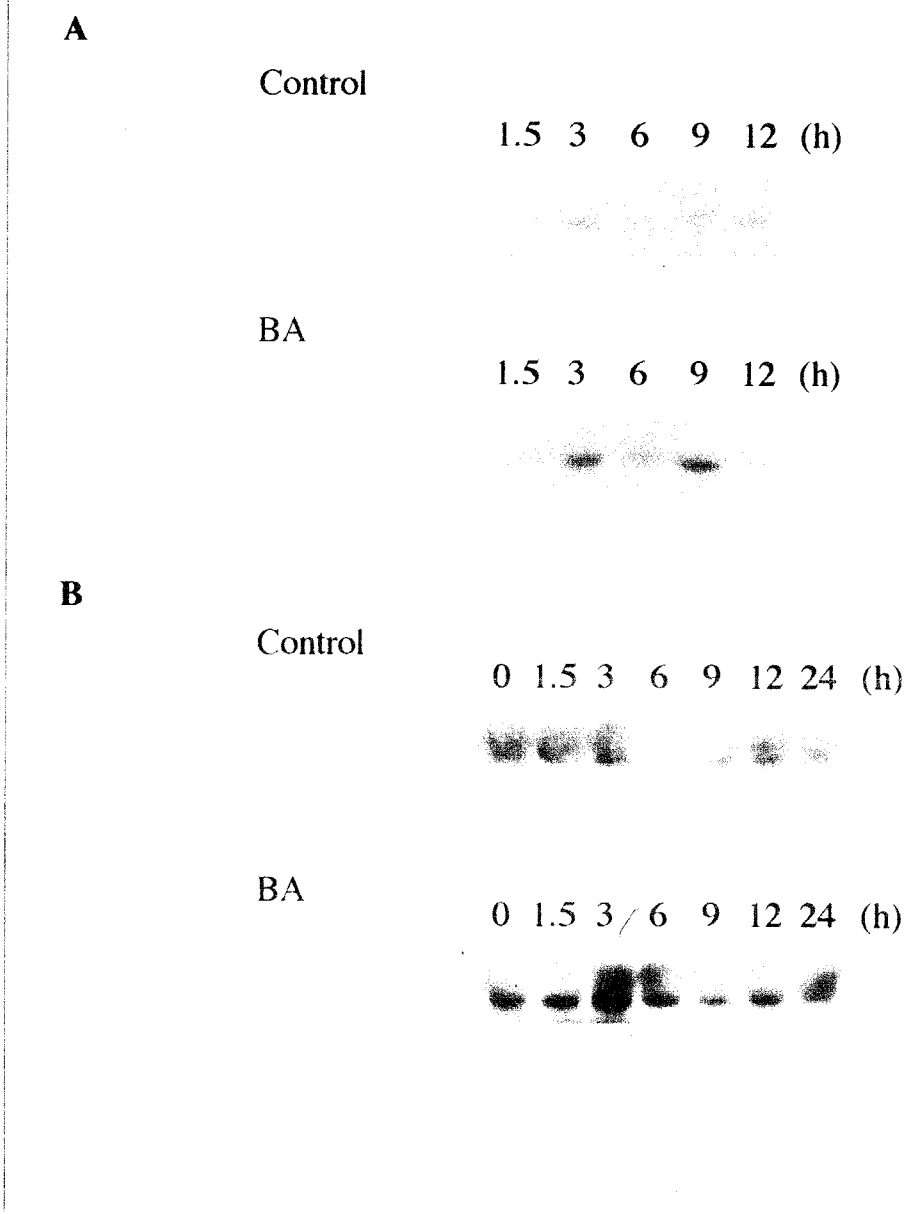


Figure III-9. Effects of light and BA on the expression of cucumber glutamyl-tRNA reductase gene. (A) Cotyledons were excised from etiolated cucumber seedlings and incubated with 50 μM BA or water in darkness. (B) Cotyledons were incubated with 50 μM BA or water for 12 h in darkness and then illuminated. Cotyledons were harvested at the indicated time, and total RNAs were isolated from cotyledons. Twenty μg of the RNA were separated on a 1.4% agarose gel and blotted onto nylon membranes. Autoradiography was carried out after hybridization with cucumber *hemA* cDNA.

Northern hybridization with the same oligonucleotide probe as described in Chapter II (Fig. III-10A). The expression level of *trnE* appeared to increase by light up to 6 h and decreased. An oligonucleotide probe complementary to the $tRNA^{Phe}$ gene of the barley chloroplast (5'-TCTCGTCTCCTGACTTTTAGGAGCACAGTGGTCAAGTTTA-3') was synthesized and used for measuring expression level of the gene for comparison. The expression level of $tRNA^{Phe}$ showed exactly the same pattern of changes in hybridization as that of $tRNA^{Glu}$ (Fig. III-10B).

BA almost doubled the transcription levels of both *trnE* and *trnF* compared with those of water control (Fig. III-11A, B). In fact, amounts of total RNAs in plastids from BA-treated and untreated cotyledons were 242 μg and 131.5 $\mu g/10^9$ plastids, respectively. These results suggested that light and BA increased the level of substrate for ALA synthesis, and is in accordance with the previous assumption that the stimulation of formation of glutamyl-tRNA is caused by BA- and light-induced increase of endogenous level of $tRNA^{Glu}$ (Table III-2).

Effect of BA and light on the levels of cofactors in stroma

Glutamate is a substrate for glutamyl-tRNA synthetase. The levels of glutamate in stroma were determined by two methods, *i.e.*, enzymatic determination and HPLC analysis of glutamate derivative. Table III-5 shows the levels of glutamate in intact plastids from greening cotyledons determined by the enzymatic analysis. Before illumination, the level of glutamate in stroma remained at low level (40-50 $nmol/10^9$ plastids). Upon illumination, the levels of glutamate increased 6-7 fold than that of before illumination, and then slightly decreased. This showed that the synthesis or uptake of glutamate in plastids could be involved in the light-induced stimulation of ALA synthesis.

BA did not cause significant change in levels of glutamate in the stromal fraction. The levels of glutamate and other amino acids in the plastids were

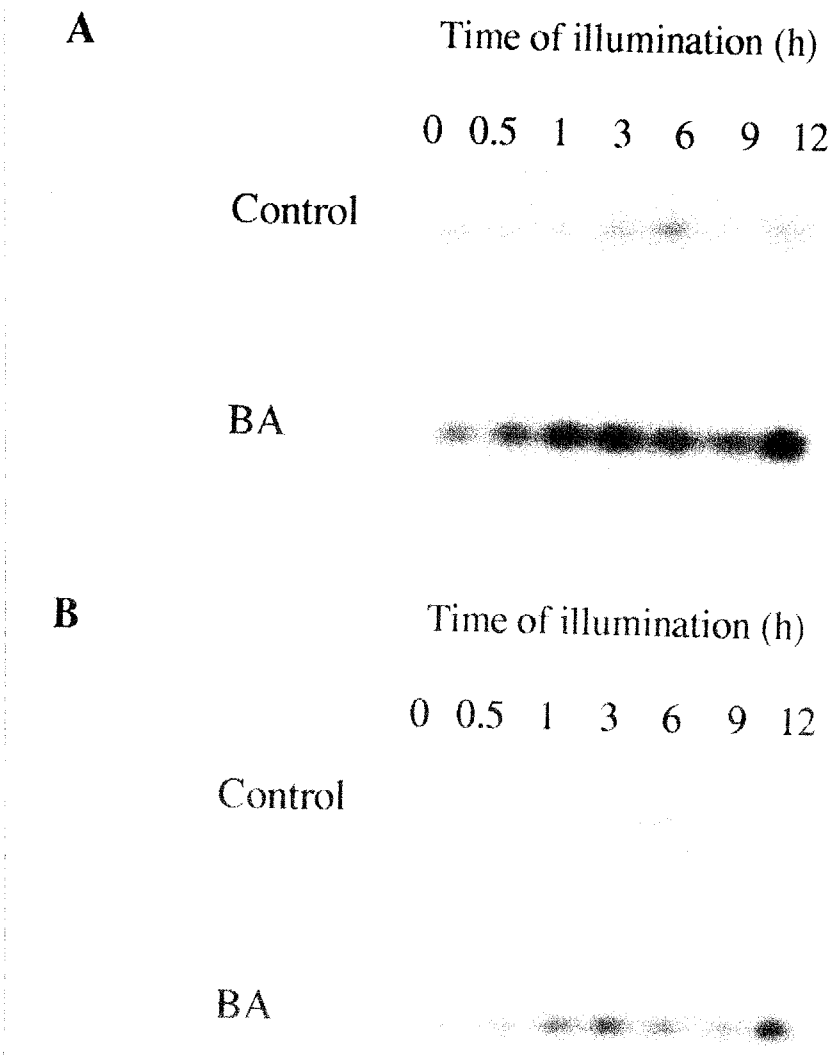


Figure III-10. Effects of light and BA on the expression of cucumber $tRNA^{Glu}$ (A) and $tRNA^{Phe}$ (B) genes. Cotyledons were excised from etiolated cucumber seedlings and incubated with 50 μM BA or water for 12 h in darkness, and then illuminated for 0.5, 1, 3, 6, 9 and 12 h. Total RNA was separated on a 4% agarose gel and blotted onto a nylon membrane. Autoradiography was carried out after hybridization with oligonucleotide probes complementary to $tRNA^{Glu}$ and $tRNA^{Phe}$ sequences.

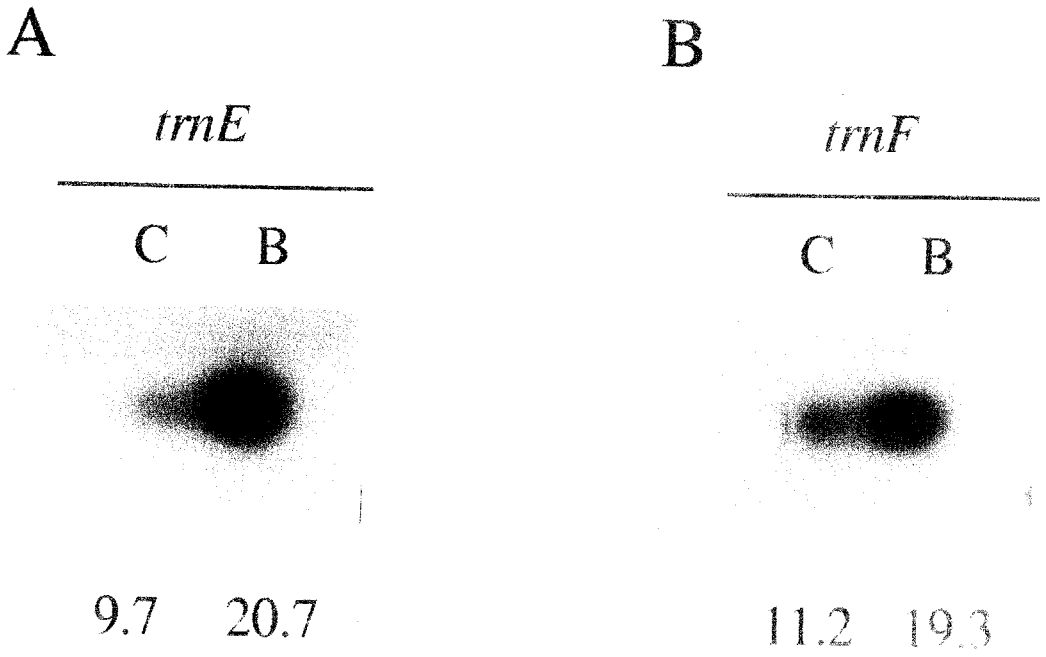


Figure III-11. Effects of BA on the levels of transcripts of *trnE* and *trnF*. Total RNA was extracted from plastids, which were obtained as described in the legend to Fig. IV-12. An aliquot (equivalent to 4 x 10^6 plastids) of the total RNA was subjected to electrophoresis on a 4% agarose gel and blotted onto a nylon membrane. Autoradiography was carried out after hybridization with oligonucleotide probes complementary to the sequence of $tRNA^{Glu}$ (A) and $tRNA^{Phe}$ (B). B, After treatment with BA; C, control. The number below each lane represents the relative radioactivity of the corresponding hybridized band, as determined with a Bioimage Analyzer.

Table III-5

The levels of glutamate in plastids from BA-treated and untreated cucumber cotyledons.

Treatment	Glutamate content (nmol/10^9 plastids)		
	Time of illumination (h)		
	0	1.5	8
Control	38	290	253
BA	51	308	243

The values are the means of results from duplicate samples.

Table III-6

The levels of ATP in plastids from BA-treated and untreated cucumber cotyledons.

Treatment	ATP content (nmol/10^9 plastids)		
	Time of illumination (h)		
	0	1.5	8
Control	1.17	2.93	7.26
BA	1.23	3.15	8.15

The values are the means of results from duplicate samples.

analyzed by the other method, HPLC. By the HPLC analysis, the level of glutamate was a little higher (319 nmol/10^9 plastids) than that (253) of enzymatic determination. BA did not cause significant changes in levels of either glutamate (363) or other amino acids in the stromal fraction.

ATP is required for the reaction of aminoacylation of glutamate with $tRNA^{Glu}$, which is catalyzed by glutamyl-tRNA synthetase. The levels of ATP increased with light illumination, which are probably due to concomitant increase in photosynthetic activity of plastids, but BA did not affect the levels of ATP (Table III-6).

Mg is required for the activity of glutamyl-tRNA synthetase. The levels of Mg in stroma were decreased by light illumination (Table III-7). In BA-treated plastids, the levels of Mg were lower than that of control.

NADPH is a cofactor for reduction of glutamyl-tRNA to generate GSA, which is catalyzed by glutamyl-tRNA reductase. BA had no effect on the levels of NADPH in plastids (Table III-8). The levels of NADPH were increased by light illumination, which is concomitant with the level of ATP, and it might be in parallel with the activity of photosynthetic light reaction but not the accumulation of GSA (Fig. III-6).

Approximate estimation showed that 10 nmol of each cofactor (*i.e.* ATP, Mg and NADPH) per 10^9 plastids correspond to the range of millimolar concentration by the assumption that inner volume of one plastid is about 10^{-12} cm^3. The range of the concentration of these cofactors, therefore, is almost about saturating one for all enzymes described above. Thus, changes of concentration of these cofactors do not affect the enzyme activities.

Table III-7

The level of Mg in plastids from BA-treated and untreated cucumber cotyledons.

Treatment	Mg content (μg/10^9 plastids)		
	Time of illumination (h)		
	0	1.5	8
Control	9.02	6.41	4.94
BA	7.35	5.39	1.52

The values are the means of results from duplicate samples.

Table III-8

The level of NADPH in plastids from BA-treated and untreated cucumber cotyledons.

Treatment	NADPH content (nmol/10^9 plastids)		
	Time of illumination (h)		
	0	1.5	8
Control	3.65	5.38	11.80
BA	2.93	5.79	9.62

The values are the means of results from duplicate samples.

DISCUSSION

Effects of light on ALA synthesis

The formation of Chl is totally dependent on light illumination. Although the synthesis of protochlorophyllide from exogenous ALA occurs even in the dark, the enzymes involved in these steps in chlorophyll biosynthesis appear to be constitutively expressed in the dark as well as in the light. Light elevated the accumulation and the rate of synthesis of ALA (Fig. III-3). It takes for about 3 h to reach the maximum rate of ALA synthesis, and it may cause the lag phase for Chl synthesis. The activity of ALA synthesis in plastids was similarly increased by light illumination (Table III-1). The substantial ability of ALA synthesis was observed even in the dark. ALA synthesis in the dark occurred only after a red-light pulse (Fig. III-2). Ford and Kasemir (1980) reported that accumulation in the dark is linearly correlated with the degree of protochlorophyllide to chlorophyllide photoconversion. The lack of ALA synthesis in the dark, without use of a light flash, is probably due to direct inhibition by the large amount of protochlorophyllide pool, although the protochlorophyllide is not as an effective feedback inhibitor as heme (Weinstein and Beale, 1985).

Glutamyl-tRNA synthetase catalyzes aminoacylation of $tRNA^{Glu}$ with glutamate, which is the first step of ALA synthesis. There is only one glutamate specific aminoacyl-tRNA synthetase in the stroma of higher plants, which activates glutamate for both protein and chlorophyll synthesis. When the endogenous $tRNA^{Glu}$ are used as substrate, the activity of glutamyl-tRNA synthetase was increased by light illumination (Table III-2). However, this increase was dependent on the level of $tRNA^{Glu}$, because when equal amount of total RNA was added to the reaction mixture after RNase A and RNase inhibitor treatment, they showed the same activity. Actually, the level and of glutamyl-tRNA synthetase was not changed by light (Fig. III-7). This is in

accordance with the results of O'Neill and Söll (1990). Thus the regulation of light on this enzyme is negligible.

Glutamyl-tRNA reductase, catalyzing the second step of ALA synthesis, is one of the most important steps from view point of regulation (Kannangara *et al.*, 1988) because heme inhibits this enzyme through feedback regulation as shown in Chapter IV (cf. Wang *et al.*, 1984). Glutamyl-tRNA reductase is an enzyme that catalyzes the NADPH-dependent reduction of Glu-tRNA^{Glu} to GSA. The knowledge for the biochemical property of this enzyme is still insufficient due to the lack of purified enzyme, which is present only in low abundance in the organisms tested (Jahn *et al.*, 1992). Gene encoding glutamyl-tRNA reductase has been isolated by complementation of the *E. coli hemA* mutation from bacteria such as *E. coli* (Drolet *et al.*, 1989) and *Salmonella typhimurium* (Eliott, 1989). In higher plants, Ilag *et al.* (1994) and Tanaka *et al.* (unpublished data) isolated the *hemA* gene by the same strategy from *Arabidopsis thaliana* and cucumber, respectively.

In this assay, I used *hemA* cDNA from cucumber as a probe. The level of the gene in the dark was almost undetectable. The expression of this gene increased with light illumination. The activity of glutamyl-tRNA reductase was also increased by light (Table III-3). The change of both the level and the activity of glutamyl-tRNA reductase seemed to match with the curve of the rate of synthesis of ALA under illumination (Fig. III-3). These results indicate that light regulates glutamyl-tRNA reductase expression, and this contributes the light-induced stimulation of ALA synthesis.

From the results of light dependent accumulation of GSA, Western blot analysis of total and stromal proteins against anti-GSA aminotransferase, and the assay of GSA aminotransferase, I concluded that the step of GSA aminotransferase is not responsible for the light-regulation of ALA synthesis. In *Euglena gracilis*, the step of GSA aminotransferase is mostly affected by

light (Mayer and Beale, 1990). Until now, *Euglena gracilis* is found to be an only organism which utilizes both Shemin pathway and C_5 pathway. The light response of *Euglena gracilis* is different from that of the higher plants and it is insensitive to phytohormones. Therefore, it is supposed that different mechanism may operate in different species.

The levels of substrates and cofactors required for the synthesis of ALA showed various changes with light illumination. Glutamate and $tRNA^{Glu}$ are substrates for the reaction catalyzed by glutamyl-tRNA synthetase which represents the first step in the synthesis of ALA in plastids. Light illumination greatly increased the level of glutamate in stroma. The level of glutamate was increased more than 7 folds within 1.5 h. Glutamate and glutamine are synthesized from ammonia and 2-oxoglutarate by reactions catalyzed by the two enzymes that are referred to glutamine synthetase/glutamate synthase cycle (Miflin and Lea 1990). Sakakibara *et al.* (1992) shows that the levels of both plastidic glutamine synthetase and glutamate synthase and those of their respective mRNAs were increased in a similar manner during illumination of etiolated maize seedlings. However, the accumulation of both enzymes reached considerable levels in the later phase of greening, and this is not in accordance with the rapid increase of glutamate in stroma. Other factor(s) may be involved in the regulation of the level of glutamate. Although it has not been determined yet, the rapid increase of glutamate may contribute to the light-induced stimulation of ALA synthesis.

$tRNA^{Glu}$ is one of the substrates for glutamyl-tRNA synthetase. Northern blot analysis showed that *trnE* was transcribed in the dark and that the expression level was certainly increased during greening after exposure to light. The pattern of changes in the expression level of $tRNA^{Glu}$ was exactly the same as that of another tRNA gene ($tRNA^{Phe}$) which is located at a distance from *trnE* in the chloroplast genome. This shows that specific changes in the expression levels of both tRNA genes were not controlled by light, because the

same amount of total RNA from cotyledons illuminated for different periods was loaded on agarose gels used for Northern blot analysis. For the reasons of non-specifically increase of tRNAs, Naito *et al.* (1979) reported that the possible increase of the chloroplast DNA copy number. Although the increase of tRNAs by light is nonspecific, it is probable that the increased amount of $tRNA^{Glu}$ contributes to the light-stimulated formation of ALA, since light-induced increase of $tRNA^{Glu}$ stimulated the formation of glutamyl-tRNA (Table III-2).

ATP and NADPH are other cofactors for ALA synthesis. In plastids, they are mainly produced by photosynthetic light reactions. The levels of both cofactors in plastids were increased by light illumination, concomitantly with photosynthetic activity of plastids. Mg, another cofactor of ALA synthesis, was decreased by light illumination. The levels of these cofactors were greatly increased or decreased on illumination. In general, these factors seemed to accelerate ALA synthesis unless these factors were formed in excess amounts. Therefore, it was possible that the increases and decreases of these factors compensated acceleration and deceleration effects although it was impossible to specify each cofactor.

Effects of BA on ALA synthesis

BA stimulated Chl and ALA accumulation during greening *in vivo* (Fig. III-1), in agreement with the results of Ford *et al.*(1979), who measured ALA synthesis in the light after a dark preincubation with BA. Treatment with BA doubled the levels of Chl and ALA synthesis. This suggests that BA stimulates the synthesis of the component(s) required for ALA synthesis.

To elucidate the effect of BA on the third step of ALA synthesis, GSA aminotransfer, GSA accumulation was determined with gabaculine. Gabaculine is a potent inhibitor of GSA aminotransferase, and it strongly inhibited chlorophyll formation in excised cotyledons (Fig. III-5). By the treatment with

gabaculine, a significant amount of GSA accumulated in treated leaves. BA treatment stimulated the GSA accumulation in gabaculine treated cotyledons. The stimulation of GSA accumulation by BA was the same degree as the stimulation of ALA accumulation *in vivo*. Both the levels and the activity of GSA aminotransferase were not affected by BA (Fig. III-8, Table III-4). Therefore, it is unlikely that the BA-induced stimulation of ALA synthesis is due to an induction of synthesis of GSA aminotransferase, and the site of action of BA might exist before the step of GSA aminotransferase.

BA increased the levels of glutamyl-tRNA reductase in the dark and the light. Actually, the activity of glutamyl-tRNA reductase was increased by BA in the dark and the light. As described before, glutamyl-tRNA reductase gene is encoded in nuclear genome. Since the levels of glutamyl-tRNA reductase were very low in the darkness in water control (Fig. III-9A), and cycloheximide inhibited the stimulation of ALA synthesis by BA in the dark and light (Fig. III-4A, B), it could be considered that BA elevates the levels of glutamyl-tRNA reductase in the dark to the level enough to reduce glutamyl-$tRNA^{Glu}$ upon illumination, and this may cause the stimulation of ALA synthesis by BA.

BA did not affect both the activities and glutamyl-tRNA synthetase. Furthermore, the levels of either glutamyl-tRNA synthetase or glutamate in stroma. BA did not increase the levels of cofactors involved in ALA synthesis, *i.e.*, ATP, NADPH and Mg.

BA increased the levels of $tRNA^{Glu}$. The synthesis of Glu-$tRNA^{Glu}$ was also doubled by BA treatment. The transcript of *trnE* was not amplified specifically because the transcript of *trnF*, which is located at a distance from *trnE* in the plastid genome, was similarly increased by BA. It has been reported that BA doubled plastid DNA copy number per chloroplast (Naito *et al.* 1979, Kinoshita and Tsuji 1984). Level of plastid rRNA is also doubled by BA (Kinoshita *et al.* 1979). Therefore, it was possible that BA increased the plastid

DNA copy number, which resulted in the increase in total plastid RNA without changes in transcriptional activity of specific genes.

The previous results showed that the expression of *trnE* and *trnF* was similarly increased by light (Fig. III-10, III-11). Light possibly increases the chloroplast DNA copy number. This suggests that BA and light may share parts in the mechanism of increasing tRNA transcripts. With *Euglena gracilis*, Mayer and Beale (1990) reported that light increased the activities of all enzymes of the ALA synthesizing system together with the level of *trnE* transcript. They also showed that GSA aminotransferase activity was increased 19-fold by light. In this respect, effects of BA and light on the ALA synthesizing system are different.

Jayabaskaran *et al.* (1990) compared levels of several chloroplast tRNAs and activities of chloroplastic and cytoplasmic aminoacyl-tRNA synthetases in green, yellow senescing and BA-treated senescing leaves of bean plants. They reported that BA increased activities of aminoacyl-tRNA synthetases of BA-untreated yellow senescing leaves. In their system, however, effect of BA on levels of tRNAs varied depending on tRNA species, and BA did not increase $tRNA^{Glu}$ level of untreated senescing leaves. In our experiments using young cucumber cotyledons, BA did not affect the level of plastidic glutamyl-tRNA synthetase in plastids, but increased the level of plastid $tRNA^{Glu}$. Naito *et al.* (1978) reported that the effects of BA on DNA and Chl contents in bean leaves were different depending on their age. The discrepancy between results of Jayabaskaran *et al.* and mine may be due to the difference in the species and in the developmental stage such as old senescing and young greening tissues.

In this Chapter, I tried to elucidate the mechanism of ALA synthesis by measuring the cellular level of each component involved in the ALA-synthesizing system in cucumber. The results indicated that cellular levels of the tRNA and the enzymes are regulated by BA and light. In Chapter V, I will

generally and conclusively discuss the mechanism of regulation of ALA synthesis in cucumber.

REFERENCES

Arnon, D. I. (1949) Copper enzymes in isolated chloroplasts: Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.*, **24**:1-15.

Adedipe, N. O., Hunt, L. A. and Fletcher, R. A. (1971) Effects of benzyladenine on photosynthesis, growth and senescence of the bean plant. *Plant Physiol.*, **25**: 151-153.

Beutler, H. O. and Michal, G. (1974) Determination with glutamate dehydrogenase, diaphorase, and tetrazolium salts. *In* Methods of Enzymatic Analysis, Vol. 4. Edited by Bergmeyer, H. U. pp. 1708-1713. Academic Press, Inc., New York.

Castelfranco, P. A., Rich, P. M. and Beale, S. I. (1974) The abolition of the lag phase in greening cucumber cotyledons by δ-aminolevulinic acid. *Plant Physiol.*, **53**: 615-618.

Dei, M. and Tsuji, H. (1987) Synthesis of 5-aminolevulinic acid by plastids isolated from cotyledons of greening cucumber seedlings pretreated with benzyladenine. *Plant Physiol. (Life Sci. Adv.)*, **6**: 63-68.

Drolet, M., Peloquin, L., Echelard, Y., Cousineau, L. and Sasarman, A. (1989) Isolation and nucleotide sequence of the *hemA* gene of *Escherichia coli* K12. *Mol. Gen. Genet.*, **216**: 347-352.

Duggan, J. and Gassman, M. (1974) Induction of porphyrin synthesis in etiolated bean leaves by chelators of iron. *Plant Physiol.*, **53**: 206-215.

Eliott, T. (1989) Cloning, gene characterization and nucleotide sequence of the *hemA-prfA* operon of *Salmonella typhimurium*. *J. Bacteriol.*, **171**: 3948-3960.

Ellis, R., Spooner, T. and Yakulis, R. (1975) Regulation of chlorophyll synthesis in the green alga *Golenkinia*. *Plant Physiol.*, **55**: 791-795.

Fletcher, R. A. (1969) Retardation of leaf senescence by benzyladenine in intact bean plants. *Planta*, **89**: 1-7.

Fletcher, R. A. and McCullagh, D. (1971) Cytokinin-induced chlorophyll formation in cucumber cotyledons. *Planta,* **101**: 88-90.

Fletcher, R. A., Teo, C. and Ali, A. (1973) Stimulation of chlorophyll synthesis in cucumber cotyledons by benzyladenine. *Can. J. Bot.*, **51**: 937-939.

Fluhr, R., Harel, E., Klein, S., and Meller, E. (1975) Control of δ-aminolevulinic acid and chlorophyll accumulation in greening maize leaves upon light-dark transitions. *Plant Physiol.*, **56**: 487-501.

Ford, M. J., Alhadeff, M. Chapman, J. M. and Black, M. (1979) A rapid and selective action of 6-benzylaminopurine on 5-aminolevulinate production in excised sunflower cotyledons. *Plant Sci. Lett.*, **16**: 799-803.

Ford, M. J. and Kasemir, H. (1980) Correlation between 5-aminolevulinate accumulation and protochlorophyll photoconversion. *Planta*, **150**: 206-210.

Gough, S. P., Kannangara, C. G., and Bock, K. (1989) A new method for the synthesis of glutamate 1-semialdehyde. Characterization of its structure in solution by NMR spectroscopy. *Carlsberg Res. Commun.*, **54**: 99-108.

Grimm, B., Smith, A. J., Kannangara, C. G. and Smith, M. (1991) Gabaculine-resistant glutamate 1-semialdehyde aminotransferase of *Synechococcus*. *J. Biol. Chem.*, **266**: 12495-12501.

Hewett, E. W. and Wareing, P. F. (1973) Cytokinins in *Populus* x *robusta* (Schneid): light effects on endogenous levels. *Planta*, **114**: 119-129.

Huang, D. D. and Castelfranco, P. A. (1990) Regulation of 5-aminolevulinic acid (ALA) synthesis in developing chloroplasts. III. Evidence for functional heterogeneity of the ALA pool. *Plant Physiol.*, **92**: 1003-1008.

Ilag, L. L., and Kumar, A. M. and Söll, D. (1994) Light regulation of chlorophyll biosynthesis at the level of 5-aminolevulinate formation in Arabidopsis. *Plant Cell*, **6**: 265-275.

Inoue, Y., Kobayashi, Y., Shibata, K. and Heber, U. (1978) Synthesis and hydrolysis of ATP by intact chloroplasts under flash illumination and darkness. *Biochim. Biophys. Acta,* **504**: 142-152.

Iwasaki, Y., Sasaki, T. and Takabe, T. (1990) Sequencing and expression of the gene that encodes a 20-kDa polypeptide of the PSI complex from cucumber cotyledon. *Plant Cell Physiol.*, **31**: 871-879.

Jayabaskaran, C., Kuntz, M., Guillemaut, P. and Weil, J. H. (1990) Variations in the levels of chloroplast tRNAs and aminoacyl-tRNA synthetases in senescing leaves of *Phaseolus vulgaris. Plant Physiol.*, **92**: 136-140.

Jahn, D., Verkamp, E. and Söll, D. (1992) Glutamyl-transfer RNA: A precursor of heme and chlorophyll biosynthesis. *Trends Biochem. Sci.*, **17**: 215-218.

Kannangara, C. G., Gough, S. P., Bruyant, P., Hoober, J. K., Kahn, A. and von Wettestein, D. (1988) $tRNA^{Glu}$ as a cofactor in δ-aminolevulinate biosynthesis: steps that regulate chlorophyll synthesis. *Trends Biochem. Sci.*, **13**: 139-143.

Kannangara, C. G. and Schouboe, A. (1985) Biosynthesis of δ-aminolevulinate in greening barley leaves. VII. Glutamate 1-semialdehyde accumulation in gabaculine treated leaves. *Carlsberg Res. Commun.*, **50**: 179-191.

Kinoshita, I., Katagiri, K. and Tsuji, H. (1979) Effects of benzyladenine and light on changes in various RNA species in etiolated cucumber cotyledons. *Plant Cell Physiol.*, **20**: 707-713.

Kinoshita, I. and Tsuji H (1984) Benzyladenine-induced increase in DNA content per chloroplast in intact bean leaves. *Plant Physiol.*, **76**: 575-578.

Klein, S., Harel, E, Neeman, E., Katz, E. and Meller, E. (1975) Accumulation of δ-aminolevulinic acid and its relation to chlorophyll synthesis and development of plastid structure in greening leaves. *Plant Physiol.*, **56**: 486-496.

Kohler, K. H., Dofler, M. and Goøring, H. (1980) The influence of light on the cytokinin content of *Amaranthus* seedlings. *Biol. Plant.*, **22**: 128-134.

Kuraishi S, Tasaki, K., Sakurai, N and Sadatoku, K. (1991) Changes in levels of cytokinins in etiolated squash seedlings after illumination. *Plant Cell Physiol.*, **32**: 585-591.

Lejeune, P., Kinet, J-M. and Bernier, G. (1988) Cytokinin fluxes during floral induction in the long-day plant *Sinapis alba* L. *Plant Physiol.*, **86**: 1095-1098.

Lew, R. and Tsuji, H. (1982) Effect of benzyladenine treatment duration on δ-aminolevulinic acid accumulation in the dark, chlorophyll lag phase abolition, and long term chlorophyll production in excised cotyledons of dark-grown cucumber seedlings. *Plant Physiol.*, **69**: 663-667.

Longo, G. P., Pedretti, M., Rossi, G. and Longo, C. P. (1981) Effect of benzyladenine on the development of plastids and microbodies in excised watermelon cotyledons. *Planta*, **145**: 209-217.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Mayer, S. M. and Beale, S. I. (1990) Light regulation of δ-aminolevulinic acid biosynthetic enzymes and tRNA in *Euglena gracilis*. *Plant Physiol.*, **94**: 1365-1375.

Miflin, S. F. and Lea, P. J. (1990) Ammonia assimilation. *In* The biochemistry of Plants Vol. 5. Edited by Miflin, B. J. pp. 169-202. Academic Press, Inc., New York.

Naito, K., Tsuji, H. and Hatakeyama, I. (1978) Effect of benzyladenine on DNA, RNA, protein, and chlorophyll contents in intact bean leaves: differential responses to benzyladenine according to leaf age. *Physiol. Plant.*, **43**: 367-371.

Naito, K., Tsuji, H., Hatakeyama, I. and Ueda, K. (1979) Benzyladenine-induced increase in DNA content per cell, chloroplast size, and chloroplast number per cell in intact bean leaves. *J. Exp. Bot.*, **30**: 1145-1151.

Nishinari, N. and Syono, K. (1980) Identification of cytokinins associated with mitosis in synchronously cultured tobacco cells. *Plant Cell Physiol.*, **21**: 383-393.

Ochiai, S. and Hase, E. (1970) Studies on chlorophyll formation in *Chlorella protothecoides*. 1. Enhancing effects of light and added δ-aminolevulinic acid, and suppressive effect of glucose on chlorophyll formation. *Plant Cell Physiol.*, **11**: 663-673.

Oh-hama, T. and Senger, H. (1975) The development of structure and function in chloroplasts of greening mutants of *Scenedesmus*. III. Biosynthesis of δ-aminolevulinic acid. *Plant Cell Physiol.*, **16**: 395-405.

O'Neill, G. P. and Söll, D. (1990) Expression of the *Synechocystis* 6803 $tRNA^{Glu}$ gene provides a functional excess of tRNA for protein and chlorophyll biosynthesis. *J. Bacteriol.*, **172**: 6363-6371.

Passonneau, J. V. and Lowry, O. H. (1974) Measurement by enzymatic cycling. *In* Methods of Enzymatic Analysis, Vol. 4. Edited by Bergmeyer, H. U. pp. 673-684. Academic Press, Inc., New York.

Sakakibara, H., Kawabata, S., Takahashi, H., Hase, T. and Sugiyama, T. (1992) Molecular cloning of the family of glutamine synthetase genes from Maize: Expression of genes for glutamine synthetase and ferredoxin-dependent glutamate synthase in photosynthetic and non-photosynthetic tissues. *Plant Cell Physiol.*, **33**: 49-58.

Sisler, E. C. and Klein, W. H. (1963) The effect of age and various chemicals on the lag phase of chlorophyll synthesis in the dark grown bean seedlings. *Plant Physiol.*, **16**: 315-322.

Sugiura, M. (1963) Promotion of chlorophyll synthesis by kinetin. *Bot. Mag. Tokyo*, **76**: 309-310.

Stetler, D. A. and Laetsch, W. M. (1965) Kinetin-induced chloroplast maturation in cultures of tobacco tissue. *Science*, **149**: 1387-1388.

Troxler, R. F. and Lester, R. (1967) Biosynthesis of phycocyanobilin. *Biochemistry*, **6**: 3840-3846.

Uheda, E. and Kuraishi S. (1977) Increase of cytokinin activity in detached etiolated cotyledons of squash after illumination. *Plant Cell Physiol.*, **18**: 481-483.

Urata, G. and Granick, S. (1963) Biosynthesis of α-aminoketones and the metabolism of aminoacetone. *J. Biol. Chem.*, **238**: 811-820.

van Staden, J. and Wareing, P. F. (1972) The effect of photoperiod on levels of endogenous cytokinins in *Xanthium strumarium*. *Plant Physiol.*, **27**: 331-337.

Wang, W. Y., Gough, S. P. and Kannangara, C. G. (1981) Biosynthesis of δ-aminolevulinate in greening barley leaves. IV. isolation of three soluble enzymes required for the conversion of glutamate to δ-aminolevulinate. *Carlsberg Res. Commun.*, **46**: 243-257.

Wang, W. Y., Huang, D. D., Stachon, D., Gough, S. P. and Kannangara, C. G. (1984) Purification, characterization and fractionation of the δ-aminolevulinic acid synthesizing enzymes from light grown *Chlamydomonas reinhardtii* cells. *Plant Physiol.*, **74**: 569-575.

Watanabe, Y. and Imai, K. (1984) Sensitive detection of amino acid in human serum and dried blood disc of 3 mm diameter for diagnosis of inborn errors of metabolism. *J. Chromatogr.*, **309**: 279-286.

Weinstein, J. D. and Beale, S. I. (1985) Enzymatic conversion of glutamate to δ-aminolevulinate formation in soluble extracts of the unicellular alga *Chlorella vulgaris*. *Arch. Biochem. Biophys.*, **237**: 454-464.

Chapter IV

A Feedback Regulation by Heme of ALA Synthesis in Cucumber

INTRODUCTION

The synthesis of ALA is regulated through a feedback regulation by its intermediates and end products. Heme is a potent inhibitor of this step in intact plastids and in most of the soluble enzyme systems that have been characterized. Exogenous heme administered to intact barley shoots (Hendry and Stobart, 1978) and to cultures of *Chlamydomonas reinhardtii* (Hoober and Stegeman, 1973) inhibited the formation of Chl *in vivo*, although in both cases the concentration of exogenous heme was extremely high. In enzyme preparations from *Chlorella vulgaris* (Weinstein and Beale, 1985), higher plants (Chereskin and Castelfranco, 1982), cyanobacteria (Rieble and Beale, 1991), and green sulfur bacteria (Rieble *et al.*, 1989), the synthesis of ALA is inhibited by heme when supplied in the range of micromolar concentration. No other tetrapyrrole intermediates or end product is as an effective inhibitor as heme (Weinstein and Beale, 1985).

It was reported that of the three enzyme activities required for ALA formation *in vitro*, heme exerts its effect only on the enzyme responsible for reduction of the glutamyl-tRNA adduct (Huang and Wang, 1986a, Wang *et al.*, 1984). However, result from separated fractions from *Chlamydomonas* revealed that both the glutamyl-tRNA synthetase and the reductase activities were inhibited by heme, with a 50% inhibition occurring at 2 and 10 μM for the synthetase and reductase steps, respectively (Lin *et al.*, 1989).

There is indirect evidence that heme inhibits ALA formation in plants. The aromatic heavy metal chelators, such as 2,2'-dipyridyl, stimulated the ALA and porphyrin formation in the greening leaves and the formation is accompanied by a fall in the heme content of the leaves (unpublished data, cf. Gassman and Duggan, 1974, Granick, 1959). 2,2'-Dipyridyl has also been reported to stimulate the incorporation of ^{14}C-glutamate into ALA and Mg-protoporphyrin IX in chloroplasts isolated from greening cucumber cotyledons (Castelfranco and Schwarcz, 1978, Weinstein and Castelfranco, 1978). Castelfranco and Jones (1975) demonstrated by radioactive labeling study that a rapid turnover of heme occurs in greening barley tissues. In view of their results, it is likely that the stimulation of ALA accumulation was due to the release of inhibition by heme, a feedback inhibitor.

Like aromatic chelators, diphenyl ether compounds (DPEs) and other peroxidizing herbicides induce accumulation of ALA and porphyrins, mainly protoporphyrin IX (Proto), in treated tissues (Lydon and Duke, 1988, Matringe and Scala, 1987, 1988, Witkowski and Halling, 1988, Kouji *et al.*, 1988, 1989). DPEs are important herbicides that have strong herbicidal activity in target weeds. DPEs have been in use since 1962 for the control of annual grasses and dicotyledonous weeds in soybean (*Glycine max*), peanut (*Arachis hypogaea*), cotton (*Gossypium*), rice (*Oryza sativa*) and other crops (Matsunaka, 1969). Like 2,2'-dipyridyl, DPEs possibly regulates ALA synthesis by feedback by heme. However, the mechanism of DPEs-induced accumulation of ALA and porphyrins is not yet fully understood.

In this chapter, first, I determined the site of heme-inhibition step in ALA synthesizing system in cucumber. Then I investigated the effects of DPEs on ALA synthesis. To avoid the effect of light on the DPEs-treated tissues, most experiments were done in the dark with etiolated cucumber cotyledons because DPEs destroys photosynthetic membranes by lipid peroxidation caused by their photodynamic action (Matringe and Scala, 1988, Witkowski and Halling, 1988).

When it is difficult to assay with the extracts from cucumber cotyledons, non-chlorophyllous tobacco cell cultures or green spinach leaves were used as other dicot materials. Acifluorfen-methyl (AFM) and Nitrofluorfen (NF) were used as DPE compounds.

MATERIALS AND METHODS

The followings were essentially the same as those described in Chapters II and III: Isolation of plastids and stromal proteins, and assays for activity of ALA-synthesizing system *in vivo*, glutamyl-tRNA synthetase, glutamyl-tRNA reductase, and GSA aminotransferase

Plant materials and growth conditions

Cucumber (*Cucumis sativus* L. cv. Okuji) seeds were germinated on wet vermiculite for 7 days in the dark at 25°C. Nonchlorophyllous cell suspension cultures of tobacco (*Nicotiana tabacum* L. cv. Samsun), originally obtained by Nakamura *et al.* (1985), were grown at 25°C at 53 $\mu E/m^2 \cdot s$, and subcultured every 9 days. Spinach (*Spinacia oleracea* L.) was grown in a field for 30 days.

Assay of overall activity of cell-free ALA-synthesizing enzymes

In cucumber, overall activity of ALA-synthesizing enzymes was carried out according to the same procedure as described in Chapter II. In tobacco cell culture, cells were collected by filtration and isolations of plastids and stromal proteins were carried out as described above. Activity of ALA-synthesizing enzymes was assayed at 29°C for 20 min in the reaction mixture containing 2 ml of the crude enzyme solution, 10 μM LA, 25 μM $MgCl_2$, 1 μM NADPH, 5 μM ATP and 200 nM L-glutamate. ALA extraction and determination were performed as described above.

Assay for ferrochelatase activity

Ferrochelatase is the key enzyme for biosynthetic pathway of heme. The activity is closely related to the formation of heme in the tissues (Jones, 1968, Little and Jones, 1976). Spinach was treated by spraying a dilute solution of an emulsion containing 0.4% AFM and 10% surfactant (Dain, Takeda Chemical Co.) in acetone. The application rate was 0.26 kg active ingredient/ha, which was usual rate in the fields. After 6 h in darkness, the leaves were collected and

the enzyme preparation from spinach leaves was carried out according to Porra and Lascelles (1968). Ten grams of the leaves were homogenized with a Waring blender.

From the reason that oxygen inhibits the ferrochelatase activity, the assays were done with an ordinary Thunberg tubes under anaerobic conditions. Each Thunberg tube contained 2.5 ml of a reaction mixture consisted of 40 μM mesoporphyrin IX, 0.1 ml ethanol, 100 μM Tris-HCl ,pH. 8.2, 4 μM GSH, 40 μM $FeSO_4$ and 0.1 ml of 1% Tween 80, separating the enzyme solution in the side arm. One drop of Antifoam A emulsion was added to the reaction mixture. The Thunberg tubes were evacuated and flushed with O_2-free N_2 gas for several times and finally sealed under a slight positive pressure of N_2. The tube was transferred to a water bath at 28°C and after 5 min the reaction was started by tipping the enzyme into the reaction mixture. Reaction was stopped by addition of 0.5 ml of pyridine, followed by addition of 0.5 ml of 1N NaOH. The difference in the spectra of the reduced-minus-oxidized pyridine hemochromogens was recorded and heme concentration was calculated.

Extraction and determination of protoheme

Protoheme was extracted by the method of Stillman and Gassman (1978) with some modifications. Tissues (1-2 g) were homogenized with 30 ml of acetone/0.1 N NH_4OH (9/1, v/v). These extracts were discarded and resultant pellets were rinsed with 20 ml of 80% acetone. Heme was extracted by suspending the residue in 20 ml of 2% HCl in acetone. A second 20 ml of the acetone-HCl extraction was combined to the first extract, and 30 ml of peroxide-free diethyl ether was added, followed by the addition of 100 ml of deionized water. After mixing, upper phase was separated and backwashed once with 50 ml of deionized water. After evaporation of the ether, the residue was dissolved in pyridine containing 1 N NaOH, and heme was determined spectroscopically by the reduced-minus-oxidized pyridine hemochromogen according to the method of Porra and Jones (1963).

Synthesis of Proto in isolated plastids

Plastids were isolated from 7-day old etiolated cucumber cotyledons by Fuesler *et al.* (1984). Cotyledons were homogenized with 3 volumes of grinding buffer (0.5 M mannitol, 1 mM $MgCl_2$, 1 mM Na_2-EDTA, 20 mM TES, pH 7.7, 10 mM HEPES, 0.2% (w/v) BSA and 4 mM DTT) in Waring blender. Homogenates were filtered through 6 layers of gauze, and centrifuged at 1,000 x*g* for 10 min at 0°C. The resultant pellets were resuspended in 15 ml of grinding buffer, and the suspension was layered on 35 ml of 45% Percoll in the same grinding buffer, in a 50-ml centrifuge tube. The tubes were centrifuged at 6,000 x*g* for 20 min at 0°C. The material visibly separated into two phases: a wide band near the top of the tube consisting of broken and damaged plastids, and a small pellet consisting of essentially intact plastids. The pellet was resuspended gently in grinding buffer and recentrifuged at 1,000 x*g* for 15 min. The resulting pellet was resuspended in the grinding buffer.

The synthesis of Proto was carried out according to Witkowski and Halling (1989). DPEs were added to 0.5 ml plastids suspension (equivalent to 2-4 mg protein) and incubated in the dark for 10 min at 30°C. Reaction was initiated by the addition of ALA (final concentration of 6 mM) and incubation was followed. After 1 h of incubation, reaction was terminated by the addition of 0.5 ml of acetone/0.1 M NH_4OH (9/1: v/v), and reaction mixture was centrifuged at 10,000 x*g* for 15 min. The resultant supernatant was measured by fluorescence spectrophotometer (Japan Spectroscopic, model FD-770) with excitation and emission wavelengths at 407 nm and 630 nm, respectively. The concentration was determined by reference to standard Proto.

Chemicals

Acifluorfen-methyl [methyl 5-(2-chloro-4-trifluoromethylphenoxy)-2-nitrobenzoate] and nitrofluorfen (2-chloro-4-trifluoromethylphenyl 4'-nitrophenyl ether) were generously gifted from Dr. Kouji. Protoporphyrin IX, 5-aminolevulinic acid, and hemin were obtained commercially from Sigma

Chemical Co. (St. Louis, MO). Synthesized GSA and GSA aminotransferase from *Synechococcus* PCC 6301 were generous gifts from Dr. C. Gamini Kannangara.

RESULTS

1. The heme-inhibition site in ALA synthesizing system

Effects of heme on activities of the three enzymes catalyzing ALA synthesis in cucumber

Since the site of heme inhibition in ALA synthesizing system in dicot has not been determined yet, the effects of heme on the activities of three enzymes catalyzing ALA synthesis (*i.e.* reactions catalyzed by glutamyl-tRNA synthetase, glutamyl-tRNA reductase and GSA aminotransferase) were examined with stromal proteins prepared from greening cucumber cotyledons. Table IV-1 shows the effects of heme on each step of ALA synthesizing system. Neither glutamyl-tRNA synthetase nor GSA aminotransferase activity was inhibited by heme to the concentration up to 10 μM. However, heme strongly inhibited the activity of glutamyl-tRNA reductase. By the addition of 5 μM heme, glutamyl-tRNA reductase activity was decreased 57% of that of control, and at 10 μM heme, almost complete inhibition was observed. The overall activity of ALA-synthesizing system was similarly inhibited by heme. By the addition of 5 μM heme, overall activity was decreased 59%. A 50% inhibition by heme occurred at 5.3 μM and 5.6 μM for the activity of glutamyl-tRNA reductase and overall activity of ALA synthesis, respectively. These results indicated that of the three enzyme activities required for ALA formation *in vitro*, heme exerts its effect only on glutamyl-tRNA reductase. Therefore, the step of glutamyl-tRNA reductase can be regarded as the heme-inhibition and the rate-limiting step in ALA synthesis.

2. Effects of DPEs on ALA synthesis and tetrapyrrole accumulation

Effects of DPEs on ALA-synthesizing activities *in vivo*

Cucumber cotyledons were pre-treated with LA which inhibits the transformation of ALA, and the amount of synthesized ALA in the AFM- and

Table IV-1

Effects of Heme on the Overall and Each Enzyme Activity of ALA-Synthesizing System *in vitro*

Treatment	Glutamyl-tRNA synthetase	Glutamyl-tRNA reductase	GSA amino-transferase	Overall ALA synthesis
	Glutamyl-tRNA formed (cpm/mg protein·h)	ALA formed (nmol/mg protein·h)	ALA formed (nmol/mg protein·h)	ALA formed (nmol/mg protein·h)
Control	10,950 (100)	0.244 (100)	4.10 (100)	0.226 (100)
+ Heme 1 μM	10,150 (93)	0.232 (95)	4.28 (105)	0.206 (92)
+ Heme 5 μM	10,160 (93)	0.104 (43)	4.09 (100)	0.091 (41)
+ Heme 10 μM	10,250 (94)	0.024 (10)	4.21 (103)	0.047 (20)

The values are the means of results from duplicate samples. The values in parentheses show % of control.

NF-treated cotyledons was measured to clarify the effects of DPEs on the activities of ALA-synthesizing enzyme system. Table IV-2 shows the effects of DPEs on ALA-synthesizing activities in cucumber cotyledons. AFM- and NF-treated samples showed about 2.4 and 1.5 fold of the ALA-synthesizing activities of untreated controls, respectively. The ratio of increase of ALA-synthesizing activities seemed to be depend on the herbicidal activities of DPEs, indicating that the increase of ALA-synthesizing activities is tightly related to the action of DPEs.

Effects of DPEs on cell-free ALA-synthesizing enzymes

Since the ALA-synthesizing system is regulated by feedback inhibition *in vivo* as mentioned before, I extracted the soluble ALA-synthesizing system from tobacco cultured cell, and examined the direct effects of DPEs on cell-free ALA-synthesizing enzymes. Because of the high activity of ALA synthesizing system from tobacco cell line, it was used for the assay. The sensitivity of tobacco cell line to DPEs has been previously reported by Kouji *et al.* (1988). As shown in Table IV-3, the activity of cell-free ALA-synthesizing enzymes was not affected by the treatment with AFM. Since DPEs-stimulated ALA accumulation in tobacco cell cultures (Kouji *et al.* 1989), the present result suggested that DPEs did not directly stimulate the activities of the ALA-synthesizing enzymes in cucumber extracts. However, it remained to be examined whether DPEs indirectly stimulates the ALA-synthesizing enzymes by stimulation or feedback inhibition of metabolite(s) of tetrapyrrole synthesis. I will describe these possibility later more in detail.

2. Effects of heme on the ALA level in DPEs-treated cotyledons

Heme but not AFM inhibits cell-free ALA synthesizing enzymes

As described in the Introduction, heme is likely to act as a feedback inhibitor in ALA synthesis system. I, therefore, firstly examined the effect of heme on the overall activity of cell-free ALA-synthesizing enzymes. As shown

Table IV-2

Effects of DPEs on ALA-synthesizing Enzyme Activity in Cucumber Cotyledons

Treatment	ALA accumulation (nmol/g fresh weight)
Control	30.1
AFM 10 μM	71.6
NF 10 μM	44.7

Table IV-3

Effects of DPEs on the Activity of Cell-Free ALA-Synthesizing Enzyme System of Tobacco Cell Culture

Treatment	Activity of ALA formation (nmol/gfw·h)[a]
Control	66.6
AFM 10 μM	59.7*
NF 10 μM	58.2*

*The values are not significant difference to control ($P < 0.05$).
a, The concentration of cell-free extracts was normalized on the basis of gram fresh weight of cotyledons.

in Table IV-4, heme inhibited the overall activity and AFM did not recover them, together with the result in Table IV-3, indicating that AFM itself had no effect on the overall activity of enzymes.

Then I checked the effect of heme on ALA accumulation induced by DPEs in cotyledons. The assay was done by measuring the effects of heme on the DPE-induced ALA-synthesizing activity in cucumber cotyledons. As shown in Table IV-5, 5 μM of heme was found to be strong enough to inhibit the stimulatory action of AFM. The inhibition of ALA-synthesizing activity was depend on the concentration of heme. This suggested that the heme abolished the stimulation by DPEs.

In the next experiment, the content of heme in DPE-treated tissues was examined. Since free protohemes which are not associated with proteins could regulate ALA synthesis (Thomas and Weinstein 1990, 1992), I determined the level of protoheme in AFM-treated tissue according to the procedure of Stillman and Gassman (1978). As shown in Fig. IV-1, the levels of protoheme in tobacco cultured cells were decreased with time by the treatment with AFM, Similarly, the heme content in AFM-treated cucumber cotyledons also decreased compared with the control (Table IV-6).

The level of heme in the cotyledons is probably determined by the balance between its formation and degradation. The decrease in the heme level, therefore, may be due to the inhibition of the formation of heme, which is mainly catalyzed by ferrochelatase. Kouji *et al.* (1988) reported that the activity of ferrochelatase in spinach leaf homogenates was not affected by the treatment of DPEs. Therefore, it was suggested that the accumulation of heme was indirectly inhibited by the action of DPEs. I further checked the effect of DPEs on the activity of ferrochelatase in DPEs-treated green spinach leaves, which have the much higher activity of ferrochelatase than cucumber cotyledons. The activity of ferrochelatase was essentially the same in both DPE-treated leaves and the control (Table IV-7).

Table IV-4

Effects of Heme on the Overall Activity of Cell-Free ALA-Synthesizing Enzyme System in Tobacco Cell Cultures

Treatment	Activity of ALA formation (nmol/gfw·h)[b]
Control	66.6
Heme 10 μM	41.4[a]
Heme 10 μM + AFM 10 μM	37.2[a]
Heme 10 μM + NF 10 μM	42.3[a]

a, No significant difference obtained by Duncan multiple range test ($P < 0.05$) was expressed as the same letters with the values.
b, same as a in Table IV-2.
Experimental conditions were the same as in Table IV-2.

Table IV-5

Effects of Heme on the ALA Accumulation Induced by the Treatment with AFM in Cucumber Cotyledons

Treatment	ALA accumulation (nmole/g fresh weight)
Control	29.9
AFM 1 μM	67.4
AFM 1 μM + Heme 1 μM	66.8
5 μM	25.8
10 μM	17.7

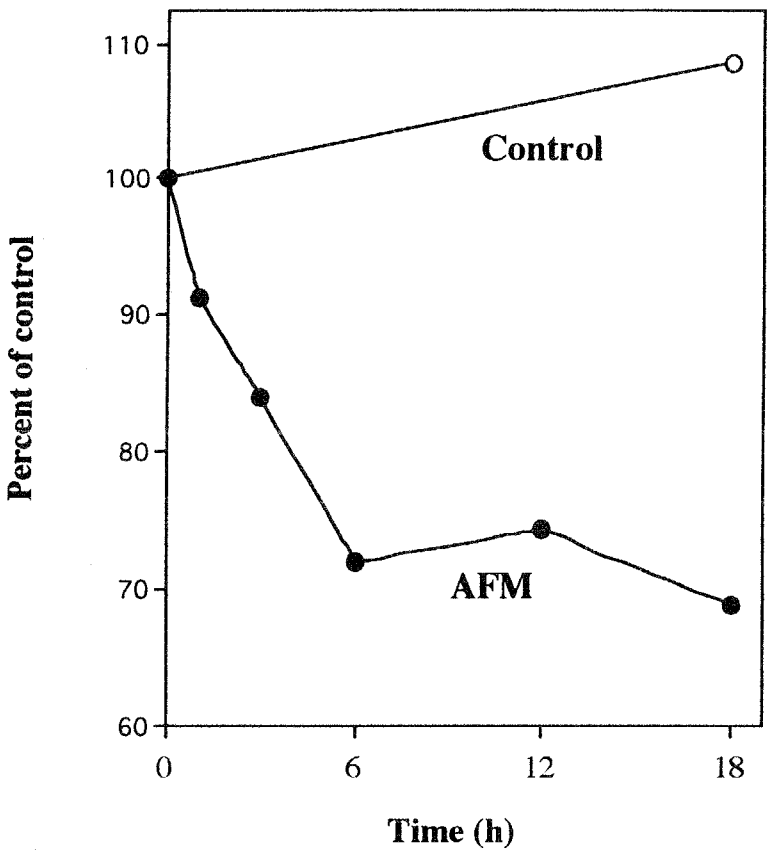


Figure IV-1. Heme content in tobacco-cultured cells treated with 1 μM AFM. Heme content: 100%=585.3 nmol/g fresh weight. ●, 1 μM AFM; ○, control.

Table IV-6

Heme Content in Etiolated Cucumber Cotyledons Treated with AFM

	Heme content (μmol/g fresh weight)	
Treatment	24 h	36 h
Control	3.1	4.2
AFM 1 μM	1.6	1.1

Table IV-7

Ferrochelatase Activity in Green Spinach Leaves Treated with AFM

	Ferrochelatase activity
Treatment	Mesoheme formed (nmol/g fresh weight·h)
Control	231±32
AFM	257±59

Note. The rate of the spray treatment was 0.26 kg active ingredient/ha. The data were obtained from five replications.

Effects of DPEs on the synthesis of protoporphyrin IX (Proto)

If DPEs inhibit the synthesis of Proto which is used for the substrate of ferrochelatase, the content of heme may decrease. Thus, the effect of DPEs on the synthesis of Proto in isolated plastids from cucumber cotyledons was examined. As shown in Fig. IV-2, AFM greatly inhibited the production of Proto *in vitro*. The inhibition of the synthesis of Proto was dependent on the concentration of AFM. At 10 μM of AFM, the inhibition of synthesis of Proto was about 80%. This showed that the site of action of AFM is located between ALA and Proto.

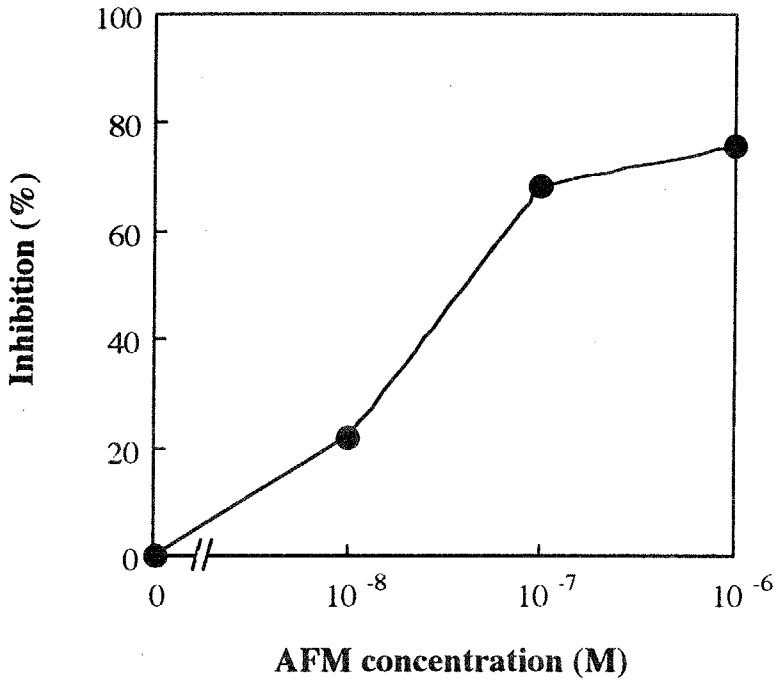


Figure IV-2. Inhibitory effect of AFM on the protoporphyrin IX synthesis in isolated plastids from etiolated cucumber cotyledons.

DISCUSSION

In this Chapter, the effects of heme on ALA synthesizing system were analyzed *in vitro* and *in vivo*. Variety of hemes function in organism as prosthetic group such as cytochromes and peroxidase. Thomas and Weinstein (1990, 1992) suggested that the hemes which are not tightly associated with proteins could act as feedback inhibitor of ALA synthesis. I examined the effects of non-protein bound protoheme on ALA synthesis in this study. The result showed that exogenously applied heme only inhibits the activity of glutamyl-tRNA reductase *in vitro*. Since the overall activity of ALA synthesis was inhibited by heme as well as glutamyl-tRNA reductase, this step can be regarded as the heme-inhibition step in ALA synthesizing system (Table IV-1). A 50% inhibition by heme occurred at 5.3 μM and 5.6 μM for the activity of glutamyl-tRNA reductase and the overall activity of ALA synthesis, respectively. This concentration is higher than that of partially purified *Chlamydomonas reinhardtii* extracts (0.05 μM; Huang and Wang, 1986b) and *Chlorella vulgaris* extracts (1.2 μM; Weinstein and Beale, 1985), but substantially lower than that of *Euglena gracilis* (25 μM; Mayer *et al.*, 1987). Since Thomas and Weinstein (1990, 1992) reported that the heme required for regulatory purposes in plastids is in the micromolar concentration range, it is likely that heme plays a significant role in regulating ALA formation from glutamate by affecting glutamyl-tRNA reductase in cucumber.

The effects of heme on *in vivo* ALA synthesis were analyzed for the elucidation of mechanism of action of DPEs. The activity of soluble ALA synthesis system from tobacco cell culture was found to be higher than that of cucumber cotyledons. The sensitivity of tobacco cell culture to DPEs is demonstrated by Kouji *et al.* (1988). Thus, as another dicot plant material, tobacco cell culture (Nakamura *et al.* 1985), which shows high activity of ALA synthesis in cell-free extracts from plastids, were used for this experiment.

Green spinach leaves were used for the assays of ferrochelatase. Although etiolated cucumber cotyledons might be an ideal plant material in experiments, green spinach leaves were used for the reason of high activity of ferrochelatase.

DPEs stimulated the accumulation of ALA in treated tissues (Table IV-2), although the extent of stimulation was not dramatic for both herbicides. The results from Table IV-3 showed that the stimulation of ALA synthesis was not due to direct stimulation of ALA-synthesizing activity by DPEs. Castelfranco and Jones (1975) suggested that the feedback inhibition by heme was the main role of the regulation of ALA synthesis.

As shown in Table IV-1, in cell-free extracts from cucumber cotyledons, heme strongly inhibited solely glutamyl-tRNA reductase but not synthetase and GSA aminotransferase. The overall synthesis of ALA-synthesizing system was also inhibited as well as glutamyl-tRNA reductase. On the other hand, however, inhibitory effect of heme on the overall synthesis activity of ALA in tobacco cell cultures was less (Table IV-4) than that in cucumber shown in Table IV-1. In this assay, I used cell-free extract from tobacco cell culture as the experimental material because of its high ALA synthesizing activity as described above. It is possible that, in cucumber, the sensitivity of heme inhibition is higher than that of tobacco cells. In fact, a 50% inhibition of overall activity of ALA synthesis occurred in 5.6 μM in cucumber, while only 40% inhibition occurred at 10 μM heme in tobacco cells. Although the effects of DPEs and heme on cell-free extract from cucumber should be examined, I could not perform this experiment because DPEs compounds were spent out and not available at the moment. However, it is obvious that DPEs did not recover the heme-inhibited activity of ALA-synthesizing system, no matter what the inhibition rate is different from cucumber and tobacco cells.

Exogenous administration of heme drastically inhibited the action of DPEs (Table IV-5) suggesting that the heme is a key substance of mechanism of action of DPEs. Thus, it could be thought that the concentration of heme in the

treated plant was deeply related to the action of DPEs. In fact, the heme content in DPE-treated tissue was decreased to half to one-third of control (Table IV-6). The extent of the decrease seems to be low. However, it is reported (Beale and Foley 1982) that the inhibition range of heme content in *Euglena gracilis* treated with *N*-methyl mesoporphyrin IX, a potent inhibitor for ferrochelatase, was about 40% compared with the control. Therefore, the results in this experiment suggested the heme synthesis was strongly suppressed.

Ferrochelatase catalyzes the insertion of Fe^{2+} ion into Proto to form heme. Kouji *et al.* (1988) demonstrated that the activity of ferrochelatase in spinach leaf homogenates was not affected by the addition of DPEs as in green leaves in the present study, It was therefore suggested that the accumulation of heme was indirectly inhibited by the action of DPEs. On the basis of these results, the indirect inhibition of ferrochelatase activity by DPEs was eliminated as the possibility on the inhibition of heme accumulation.

The stimulation of heme degradation by DPEs could be considered as another possibility on the inhibition of heme accumulation. If DPEs stimulate the further metabolism of heme, the heme content may decrease in DPEs-treated tissues. The enzyme, heme oxygenase is known to catalyze this reaction. In the present experiment the effect of DPEs on heme oxygenase was not determined. Therefore, the possibility of the stimulation of heme degradation by DPEs can not be excluded.

The results in Fig. IV-2 showed that DPE at 1 μM inhibited the synthesis of Proto drastically in the isolated plastids indicating that the formation of heme was blocked, and is consistent with Matringe *et al.* (1989a, b) who reported that DPEs strongly inhibit protoporphyrinogen oxidase that catalyzes the transformation of protoporphyrinogen to Proto.

In conclusion, a model for the feedback regulation of ALA biosynthesis by heme was presented from this work with special reference to the action of

DPEs (Fig. IV-3). The previous investigators' results support that the inhibition of protoporphyrinogen oxidase is the first target of the action of DPEs. The inhibition of protoporphyrinogen oxidase may block the direct formation of heme in plastids.

The molecular target of DPEs is very specific to the biosynthetic pathway of Chl, when compared to that of iron chelators, and this allows DPEs as useful compounds for the investigation of Chl biosynthesis. In particular, it is suggested that Chl precursor, heme, or other porphyrin could act as the signal between chloroplast and nucleus (Susek *et al.* 1993, Susek and Chory 1992). This signal could play an important role in the regulatory network by helping to coordinate chloroplast and mitochondrial functions. There is a precedent for the assumption on regulating function of porphyrins. For example, Chl precursors specifically inhibit *CAB* mRNA accumulation in *Chlamydomonas* (Johanningmeier and Howell 1984). Thus, like aromatic iron chelators, DPEs could become an effective tool for such a investigation of the role for Chl precursors.

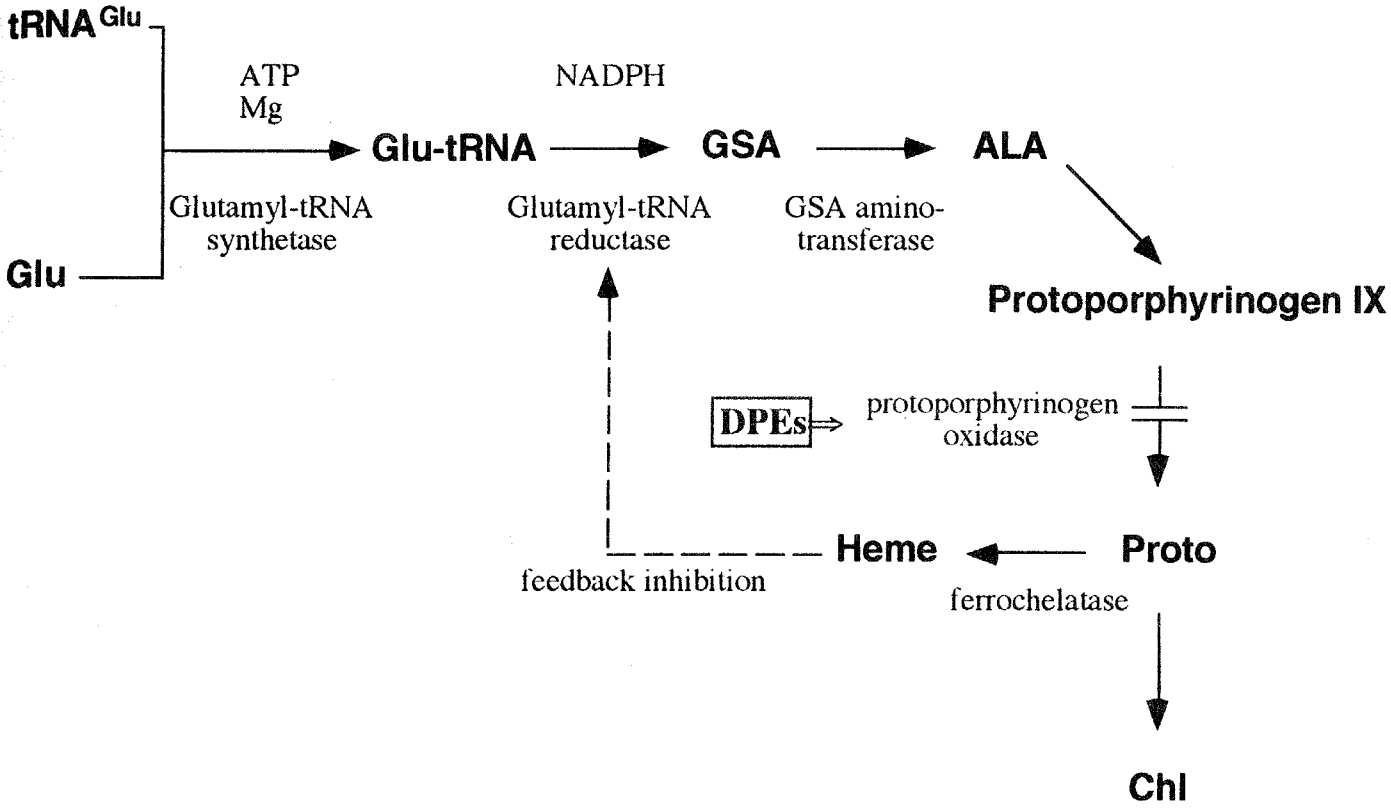


Figure IV-3. Feedback regulation of ALA synthesis by heme in cucumber. DPEs inhibit protoporphyrinogen oxidase. The inhibition of protoporphyrinogen oxidase then blocks the direct formation of heme in plastids and the decrease in heme causes the release of inhibition of glutamyl-tRNA reductase.

REFERENCES

Beale, S. I. and Foley, T. (1982) Induction of 5-amino-levulinic acid synthase activity and inhibition of heme synthesis in *Euglena gracilis* by N-methyl mesoporphyrin IX. *Plant Physiol.*, **69**: 1331-1333.

Castelfranco, P. A. and Jones, O. T. G. (1975) Protoheme turnover and chlorophyll synthesis in green barley tissue. *Plant Physiol.*, **55**: 485-490.

Castelfranco, P. A. and Schwarcz, S. (1978) Mg protoporphyrin IX and δ-aminolevulinic acid synthesis from glutamate in isolated greening chloroplast. I. Mg protoporphyrin IX synthesis. *Arch. Biochem. Biophys.*, **186**: 365-375.

Chereskin, B. A. and Castelfranco, P. A. (1982) Effects of iron and oxygen on chlorophyll biosynthesis. II. Observations on the biosynthetic pathway in isolated etiochloroplasts. *Plant Physiol.*, **69**: 112.

Fuesler, T. P., Wong, Y. S. and Castelfranco, P. A. (1984) Localization of Mg-chelatase and Mg-protoporphyrin IX monomethyl ester (oxidative) cyclase activities within isolated, developing cucumber chloroplasts. *Plant Physiol.*, **75**: 662-664.

Gassman, M. L. and Duggan, J. (1974) Chemical induction of porphyrin synthesis in higher plants. In *Proceedings of the Third International Congress on Photosynthesis*, ed. M. Avron, **3**: 2105-2113.

Granick, S. (1959) Magnesium porphyrins formed in barley seedlings treated with delta-aminolevulinic acid. *Plant Physiol.*, **34S**: xviii.

Hendry, G. A. and Stobart, A. K. (1978) The effect of haem on chlorophyll synthesis in barley leaves. *Phytochemistry*, **17**: 73.

Hoober, J. K. and Stegeman, W. J. (1973) Control of the synthesis of a major polypeptide of chloroplast membranes in *Chlamydomonas reinhardtii*. *J. Cell Biol.*, **56**: 1-12.

Huang, D. D. and Wang, W. Y. (1986a) Chlorophyll synthesis in *Chlamydomonas* starts with the formation of glutamyl-tRNA. *J. Biol. Chem.*, **261**: 13451-13455.

Huang, D. D. and Wang, W. Y. (1986b) Genetic control of chlorophyll biosynthesis: Regulation of *delta*-aminolevulinate synthesis in *Chlamydomonas*. *Mol. Gen. Genet.*, **205**: 217-220.

Johanningmeier, U. and Howell, S. H. (1984) Regulation of light-harvesting chlorophyll-binding protein mRNA accumulation in *Chlamydomonas reinhardtii*. *J. Biol. Chem.*, **259**: 13541-13549.

Jones, O. T. G. (1968) Ferrochelatase of spinach chloroplasts. *Biochem. J.*, **107**: 113-119.

Kouji, H., Masuda, T. and Matsunaka, S. (1988) Action mechanism of diphenyl ether herbicides; light-dependent O_2-consumption in diphenyl ether treated tobacco cell homogenate. *J. Pestic. Sci.*, **13**: 495-499.

Kouji, H., Masuda, T. and Matsunaka, S. (1989) Action mechanism of diphenyl ether herbicides; stimulation of 5-aminolevulinic acid-synthesizing system activities. *Pestic. Biochem. Physiol.*, **33**: 230-238.

Lin, J. M., Wegmann, B., Chang, T. N. and Wang, W. Y. (1989) The mechanism of heme inhibition of Glu-ligase. *Plant Physiol.*, **89**: 50.

Little, H. N. and Jones, O. T. G. (1976) The subcellular localization and properties of the ferrochelatase of etiolated barley. *Biochem. J.*, **156**: 309-314.

Lydon, J. and Duke, S. O. (1988) Porphyrin synthesis is required for photobleaching activity of the *p*-nitrosubstituted diphenyl ether herbicides. *Pestic. Biochem. Physiol.*, **31**: 74-83.

Matringe, M. and Scalla, R. (1987) Induction of tetrapyrroles by diphenylether type herbicides. *Proc. Brit. Crop Prot. Conf.*, **B9**: 981-988.

Matringe, M. and Scalla, R. (1988) Studies on the mode of action of acifluorfen-methyl in nonchlorophyllous soybean cells. *Plant Physiol.*, **86**: 619-622.

Matringe, M., Camadro, J. M., Labbe, P. and Scalla, R. (1989a) Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. *Biochem. J.*, **260**: 231-235.

Matringe, M., Camadro, J. M., Labbe, P. and Scalla, R. (1989b) Protoporphyrinogen oxidase inhibition by three peroxidizing herbicides: Oxadiazon, LS-82-556 and M&B 39279. *FEBS Lett.*, **245**: 35-38.

Matsunaka, S. (1969) Acceptor of light energy in photoactivation of diphenylether herbicides. *J. Agric. Food Chem.*, **17**: 171-175.

Mayer, S. M., Weinstein, J. D. and Beale, S. I. (1987) Enzymatic conversion of glutamate to δ-aminolevulinate in soluble extracts of *Euglena gracilis*. *J. Biol. Chem.*, **262**: 12541-12549.

Nakamura, C., Nakata, M., Shioji, M. and Ono, H. (1985) 2,4-D resistance in a tobacco cell culture variant: Cross-resistance to auxins and uptake, efflux and metabolism of 2,4-D. *Plant Cell Physiol.*, **26**: 271-280.

Porra, R. J. and Jones, O. T. G. (1963) Studies on ferrochelatase. 1. Assay and properties of ferrochelatase from a pig-liver mitochondrial extract. *Biochem. J.*, **87**: 181-185.

Porra, R. J. and Lascelles, J. (1968) Studies on ferrochelatase: The enzymatic formation of haem in proplastids, chloroplasts and plant mitochondria. *Biochem. J.*, **108**: 343-345.

Rieble, S. and Beale, S. I. (1991) Purification of glutamyl-tRNA reductase from *Synechocystis* sp. PCC 6803. *J. Biol. Chem.*, **266**: 9740-9745.

Rieble, S., Ormerod, J. G., and Beale, S. I. (1989) Transformation of glutamate to δ-aminolevulinic acid by soluble extracts of *Chlorobium vibrioforme*. *J. Bacteriol.*, **171**: 3782-3787.

Stillman, L. C. and Gassman, M. S. (1978) Protoheme extraction from plant tissue. *Anal. Biochem.*, **91**: 166-172.

Susek, R. E. , Ausubel, F. M. and Chory, J. (1993) Signal transduction mutants of *Arabidopsis* uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. *Cell*, **74**: 787-799.

Susek, R. E. and Chory, J. (1992) A tale of two genomes: role of a chloroplast signal in coordinating nuclear and plastid genome expression. *Aust. J. Plant Physiol.*, **19**: 387-399.

Thomas, J. and Weinstein, J. D. (1990) Measurement of heme efflux and heme content in isolated developing chloroplasts. *Plant Physiol.*, **94**: 1414-1423.

Thomas, J. and Weinstein, J. D. (1992) Free heme in isolated chloroplasts: an improved method of assay and its physiological importance. *Plant Physiol. Biochem.*, **30**: 285-292.

Wang, W. Y., Huang, D. D., Stachon, D., Gough, S. P. and Kannangara, C. G. (1984) Purification, characterization, and fractionation of the 5-aminolevulinic acid synthesizing enzymes from light-grown *Chlamydomonas reinhardtii* cells. *Plant Physiol.*, **74**: 569-575.

Weinstein, J. D. and Castelfranco, P. A. (1978) Mg protoporphyrin IX and δ-aminolevulinic acid synthesis from glutamate in isolated greening chloroplasts. II. δ-Aminolevulinic acid synthesis . *Arch. Biochem. Biophys.*, **186**: 376-382.

Weinstein, J. D. and Beale, S. I. (1985) Enzymatic conversion of glutamate to δ-aminolevulinate in soluble extracts of the unicellular green alga, *Chlorella vulgaris*. *Arch. Biochem. Biophys.*, **237**: 454-464.

Witkowski, D. A. and Halling, B. P. (1988) Accumulation of photodynamic tetrapyrroles induced by acifluorfen-methyl. *Plant Physiol.*, **87**: 632-637.

Witkowski, D. A. and Halling, B. P. (1989) Inhibition of plant protoporphyrinogen oxidase by the herbicides acifluorfen-methyl. *Plant Physiol.*, **90**: 1239-1242.

Chapter V

Genaral Discussion and Conclusion

Regulation of ALA synthesis

In this study, I tried to elucidate the regulation of ALA synthesis in cucumber. The results indicated that cellular levels of the tRNA and the activities of enzymes are regulated by light and BA (Chapter III). The synthesis of ALA was also regulated by feedback inhibition (Chapter IV). In this Chapter, the mechanism of regulation of ALA synthesis is discussed as a whole. Because of no effect on GSA aminotransferase by light, BA (Chapter III) and heme (Chapter IV), and ubiquitous existence and relatively high activity of this enzyme, the step of GSA aminotransferase was excluded as the site of regulation in ALA synthesis. Accordingly, with respect to the regulation of ALA-synthesizing system, the following two steps are considered.

Step 1: Synthesis of glutamyl-tRNA

Glutamyl-tRNA synthetase is the catalyzing enzyme, and glutamate and $tRNA^{Glu}$ are the substrates of this step. The levels of glutamyl-tRNA synthetase were not changed by light and BA. Furthermore, glutamyl-tRNA synthetase was not inhibited by heme, a feedback inhibitor, as described in Chapter IV. The changed levels of cofactors, ATP and Mg, had no influence.

The content of glutamate in stroma was rapidly increased by light. This increase seemed to be much more faster than the increase of expression of plastidic glutamate generating system (Sakakibara *et al.*, 1992). Since, Klein and Mullet (1987) suggested that rapid increase in amino acids incorporation into plastids occurred after light illumination, it is likely that this increase is caused by the incorporation of glutamate into plastids. BA did not affect the level of glutamate.

The level of $tRNA^{Glu}$ was certainly increased by light, but the substantial level of $tRNA^{Glu}$ was already existed in tissue. BA increased the level of both $tRNA^{Glu}$ and glutamyl-tRNA. The level of $tRNA^{Glu}$ seemed to be increased during dark incubation by BA, and this increase seems nonspecific because another tRNA, $tRNA^{Phe}$, was similarly increased by BA. With respect to the increase of plastidic tRNAs, the increase of plastid DNA copy number was considered as described in Chapter III. Bendich (1987) suggested that the induction of plastid transcription during chloroplast biogenesis could result from increased DNA template levels. However, so far, the mechanism of replication of plastid DNA is not understood at all, besides it is regulated by a nuclear-derived factor (Wu *et al.*, 1989).

Plastid transcription activity is also determined by the level of RNA polymerase in plastids. The RNA polymerase isolated from chloroplast is a large multisubunit enzyme (Smith and Bogorad, 1974, Tewari and Goel, 1983). This enzyme contains three chloroplast-encoded subunits. Therefore, the induction of plastid RNA polymerase levels requires the synthesis of plastid-encoded subunits. Since $tRNA^{Glu}$ is transcribed by plastid RNA polymerase, and chloramphenicol had relatively low inhibitory effect on BA-induced stimulation of ALA synthesis, the level of $tRNA^{Glu}$ may not be a critical parameter for determination of the rate of synthesis of ALA. Therefore, it seemed unlikely that the synthesis of glutamyl-tRNA was the rate-limiting step of ALA synthesis. However, since chloramphenicol certainly inhibited ALA synthesis in the light, it is possible that the elevation of substrate level by light (glutamate) and BA ($tRNA^{Glu}$) contributes to the stimulation of ALA synthesis.

Step 2: Reduction of glutamyl-tRNA

Glutamyl-tRNA reductase is the enzyme that catalyzes the NADPH-dependent reduction of glutamyl-tRNA to GSA. The levels of NADPH was enhanced by light but not by BA. The activity of glutamyl-tRNA reductase is

severely inhibited by the treatment of heme (Chapter IV). Furthermore, it is present only in low abundance in the organism tested (Jahn *et al.*, 1992). The expression of glutamyl-tRNA reductase gene was light-inducible (Fig. III-9). BA increased the level of glutamyl-tRNA reductase mRNA in the dark and light. The gene encoding glutamyl-tRNA reductase is nuclear coding. The fact that cycloheximide inhibited the stimulation of ALA synthesis suggested that some nuclear gene-encoded products was involved in the light- and BA-mediated stimulation of ALA synthesis. Glutamyl-tRNA reductase is one of the cycloheximide-sensitive candidates.

Since there is only one gene for $tRNA^{Glu}$ in cucumber chloroplast genome (Chapter II, cf. Berry-Lowe, 1987, Ohyama *et al.*, 1986, Shinozaki *et al.*, 1986), $tRNA^{Glu}$ must be used for the synthesis of both protein and ALA biosynthesis. The partition of flow of glutamyl-tRNA into both synthesis is unanswered question. The partition of flow of glutamyl-tRNA can be determined by the relative concentrations of elongation factor Tu and glutamyl-tRNA reductase, which compete for binding glutamyl-tRNA. Although the level of elongation factor Tu is unknown, the step of glutamyl-tRNA reductase may be the rate-limiting step for the ALA synthesis in this sense.

Therefore, it is concluded, from the results described above, that glutamyl-tRNA reductase is the regulatory, and the rate-limiting enzyme of ALA-synthesizing system.

Feedback regulation by heme

The synthesis of ALA was regulated through a feedback inhibition. Heme is a potent inhibitor of this step. Actually, exogenously applied heme inhibited the ALA synthesis *in vivo* in cucumber cotyledons. In cucumber, heme exerted only the step of glutamyl-tRNA reductase (Chapter IV). The concentration of 50% inhibition of glutamyl-tRNA reductase and overall

activity of ALA synthesis was about 5 μM, and was consistent with other organisms reported so far.

The indirect evidence that heme inhibits ALA formation was demonstrated by the action of DPEs. DPEs inhibited the formation of protoporphyrin IX, which is the precursor of protoheme, causing decrease of protoheme content, and stimulated ALA synthesis. The action of DPEs was similar to that of aromatic iron chelators like 2,2'-dipyridyl. However the molecular target of DPEs seemed more specific than that of 2,2'-dipyridyl, because DPEs inhibit only protoporphyrinogen oxidase.

Proposed mechanism

By the facts that light elevates the endogenous level of cytokinins in tissues (Chapter III, Introduction), light and cytokinins share part of regulation of ALA synthesis. Since red and far-red light reversely affect ALA synthesis (Klein *et al.*, 1977), phytochrome must be the receptor of light, while the receptor of cytokinin has not been identified yet. Concerning the pathway of signal transduction, Bowler *et al.* (1994) suggested that the signal transduction pathway from phytochrome involves GTP-binding protein, calcium and calmodulin, but the overall pathway of signal transduction in higher plants has still not been fully understood.

Consequently, light and BA had pleiotropically affect the ALA synthesizing system. The mechanism of regulation of ALA synthesis was proposed in Fig. V-1. Since, in higher plants, there were few studies on the effects of light and hormones, and feedback regulation on the ALA synthesis, the present study will provide a significant understanding of the mechanism of regulation of ALA synthesis.

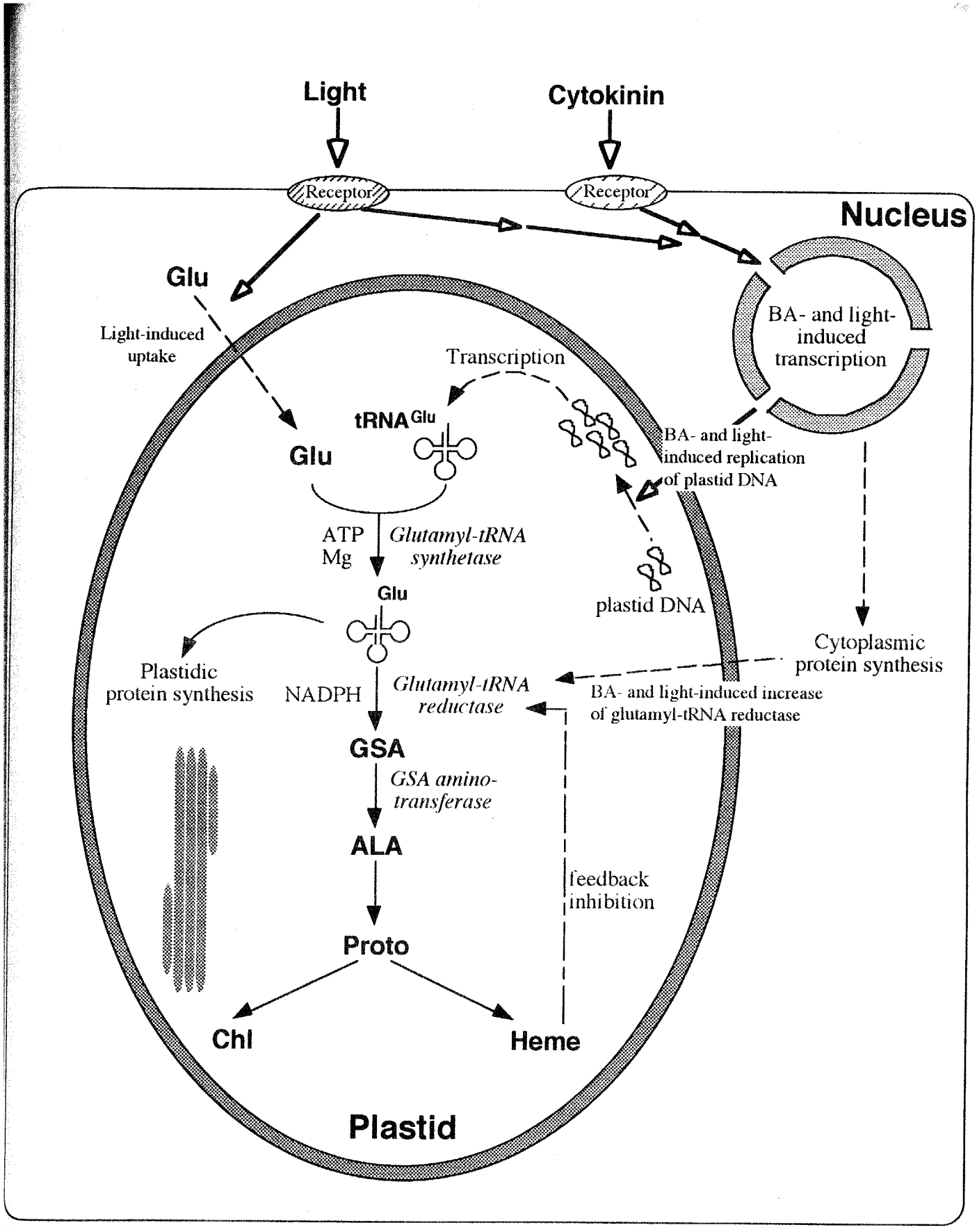


Figure V-1. Possible mechanism of regulation of ALA synthesis. In this model, metabolic pathway is indicated by solid line with arrow (◄—). The names of metabolites and enzymes are shown by bold and italic letters, respectively. The cytokinin- and light-mediated pathway are indicated by line with open arrow (◁—), and resulting effects are shown by dashed line with arrow head (◄– –).

REFERENCES

Bendich, A. J. (1987) Why do chloroplasts and mitochondria contain so many copies of their genome? *BioEssays*, **6**: 279-282.

Berry-Lowe, S. (1987) The chloroplast glutamate tRNA gene required for δ–aminolevulinate synthesis. *Carlsberg Res. Commun.*, **52**: 197-210.

Bowler, C., Neuhaus, G., Yamagata, H. and Chua, N. H. (1994) Cyclic GMP and calcium mediate phytochrome phototransduction. *Cell*, **77**: 73-81.

Jahn, D., Verkamp, E. and Söll, D. (1992) Glutamyl-transfer RNA: A precursor of heme and chlorophyll biosynthesis. *Trends Biochem. Sci.*, **17**: 215-218.

Klein, R. R. and Mullet, J. E. (1987) Control of gene expression during higher plant chloroplast biogenesis. *J. Biol. Chem.* **261**: 11138-11145.

Klein, S., Katz, E. and Neeman, E. (1977) Induction of δ-aminolevulinic acid formation in etiolated maize leaves controlled by two light reactions. *Plant Physiol.* **60**: 335-338.

Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H. and Ozeki, H. (1986) Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. *Nature*, **322**: 572-574.

Sakakibara, H., Kawabata, S., Takahashi, H., Hase, T. and Sugiyama, T. (1992) Molecular cloning of the family of glutamine synthetase genes from Maize: Expression of genes for glutamine synthetase and ferredoxin-dependent glutamate synthase in photosynthetic and non-photosynthetic tissues. *Plant Cell Physiol.*, **33**: 49-58.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B. Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H.

and Suriura, M. (1986) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. *EMBO J.*, **5**: 2043-2049.

Smith, H. J. and Bogorad, L. (1974) The polypeptide subunit structure of the DNA-dependent RNA polymerase of *Zea mays* chloroplast. *Proc. Natl. Acad. Sci. USA*, **71**: 4839-4842.

Tewari, K. K. and Goel, A. (1983) Solubilization and partial purification of RNA polymerase from pea chloroplasts. *Biochem.*, **22**: 2142-2148.

Wu, M., Nie, Z. Q. and Yang, J. (1989) The 18-kD protein that binds to the chloroplast DNA replicative origin is an iron-sulfur protein related to a subunit of NADH dehydrogenase. *Plant Cell*, **1**: 551-557.

Acknowledgments

I wish to express my sincere gratitude to Dr. Ken-ichiro Takamiya, Professor of Tokyo Institute of Technology, for his supervision and stimulating discussions. I also wish to express my acknowledgment to Dr. Yuzo Shioi, Associate Professor of Tokyo Institute of Technology, for invaluable discussion and for the support of this study.

I deeply grateful to Dr. Hideo Tsuji, Professor of Kyoto University, for his kind guidance and advices. I thank Dr. Shooichi Matsunaka, Professor of Kansai University, for valuable comments and criticisms. I am indebted to Dr. Hachiro Inokuchi, Associate Professor of Kyoto University, for his kind guidance and valuable suggestions.

I wish to thank Drs. Hiroyuki Kouji, Hiroyuki Ohta, Ayumi Tanaka for their helpful advice and valuable technical assistance. I would like to thank Dr. C. Gamini Kannangara for valuable advice and providing me with the antibodies against ALA synthesizing enzymes.

I want to express my thanks to Mr. Ryoichi Tanaka for his kind assistance. Thanks are also due to colleagues for their helpful encouragement.