STUDIES ON TRANSPORT OF THE NUCLEUS-ENCODED 30 kDa PROTEIN OF PHOTOSYSTEM II INTO *EUGLENA* CHLOROPLASTS

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CONTENTS

ABBREVIATIONS

cDNA	complementary DNA
ER	endoplasmic reticulum
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
LHC II	light-harvesting chlorophyll protein of photosystem II
OEC30	the oxygen-evolving complex 30 kDa protein of *Euglena*
PBGD	porphobilinogen deaminase
PCR	polymerase chain reaction
preOEC30	precursor of the OEC30
PS II	photosystem II
PVDF	poly(vinylidene difluoride)
Rubisco	ribulose 1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electroproresis
SSPE	a solution containing 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA (pH 7.4)
SSU	small subunit of Rubisco
TE	a solution containing 10 mM Tris-HCl and 1 mM EDTA (pH 8.0)

INTRODUCTION

Both mitochondria and chloroplasts are semi-autonomous organelles. Chloroplasts (Ellis, 1981), like mitochondria (Douglas *et al.*, 1986, Pfanner *et al.*, 1988) possess their own DNA, which encodes approximately 10% of their protein. The all remaining proteins are encoded in the nucleus. Coordinated expression of the nuclear and chloroplast genomes is required for the biogenesis of chloroplasts (Ellis, 1981, Rochaix and Erickson, 1988). Nuclear encoded proteins destined for the chloroplast are synthesized on ribosomes in the cytoplasm as the precursors (Schmidt and Mishkind, 1986). Consequently, selective targeting of newly synthesized chloroplast proteins to the chloroplasts and translocation across the chloroplast envelope membranes and/or thylakoid membrane must take place for the proteins to correctly reach their functional location in chloroplast. There are six distinct compartments within chloroplasts. These compartments are the three membranes (inner and outer envelope membrane and thylakoid membrane), stromal space, thylakoid lumen and the intermembrane space between the outer and inner envelope (Smeekens and Weisbeek, 1988).

Protein targeting to the chloroplast and translocation across and into biological membranes are the principal problems in the biogenesis of chloroplast. In most cases, nuclear encoded chloroplast precursor proteins carry specific 'targeting signals' at amino terminal extentions that are designated as transit peptides (de Boer and Weisbeek, 1991, Keegstra, 1989, Smeekens and Weisbeek, 1988). The length of the transit peptide varies from 30 to 90 amino acids, depending on protein, plant species from which the protein is obtained, and the location of the protein in chloroplast (Schmidt and Mishkind, 1986).

The transit peptides of the precursors of stromal proteins have the information necessary for binding, which is mediated by receptor proteins on the envelope membrane (Douglas *et al.*, 1986, Schmidt and Mishkind, 1986), to the chloroplast surface, translocation across the envelope membrane and targeting to the stromal compartment (Schmidt and Mishkind, 1986, Smeekens *et al.*, 1986).

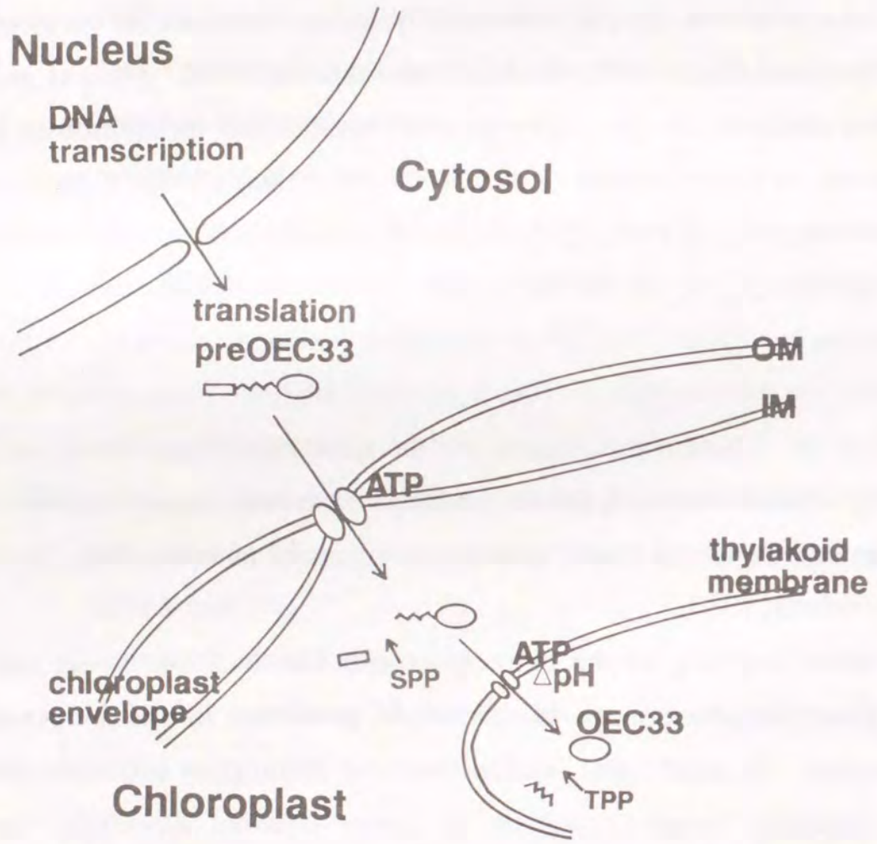

Fig. 1. Model for translocation of preOEC33 to chloroplasts. PreOEC33 is synthesized in the cytosol with bipartite presequence containing an envelope transit (a rectangle) and thylakoid transfer domain (notches) in tandem. The precursor is imported into the stroma, and is processed to intermediate form by an SPP. The thylakoid transfer domain then direct translocation across the thylakoid membrane. Translocation of OEC33 requires at least one stromal factor, ATP and a delta pH. After translocation is carried out, the intermediate form is processed to the mature size by a TPP. SPP, stromal processing peptidase ; TPP, thylakoidal processing peptidase ; OM, outer envelope membrane ; IM, inner envelope membrane.

Import into other compartments of the chloroplast, such as the thylakoid lumenal space, appears to be more complex (Fig. 1). Thylakoid lumenal proteins such as plastcyanin or the subunits of the oxygen-evolving complex must transverse three distinct membranes to reach the lumen space. The preseqences of these precursor proteins consist of two functional domains which are required for translocation across the envelopes of chloroplasts (envelope transfer domain) and across thylakoid membranes (thylakoid transfer domain) (Bassham *et al.*, 1991, Hageman *et al.*, 1990, Ko and Cashmore, 1989). The envelope transfer domain that directs import across the envelope into the stroma is hydrophilic, basic and enriched in hydroxylated amino acid residues. This domain is equivalent to the presequences of imported stromal proteins in its structure and function, and it is removed by a stromal processing protease (Hageman *et al.*, 1986, Ko and Cashmore, 1989). The thylakoid transfer domain that directs transport of the resulting stromal intermediate across the thylakoid membrane into the lumen (Hageman *et al.*, 1986, Smeekens *et al.*, 1986) is homologous to the signal sequence (Smeekens and Weisbeek, 1988) that is required for the translocation of proteins across the ER and across the plasma membrane of bacteria (von Heijne *et al.*, 1989). It contains a positively charged N-terminal region, a hydrophobic domain in the central region and a signal peptidase-processing motif at the C terminus, and it is removed by a thylakoidal processing protease (Hageman *et al.*, 1986, von Heijne *et al.*, 1989). Numerous studies have demonstrated that the transit peptide contains the information necessary to direct proteins to the chloroplast and mediate protein import and translocation (Lubben and Keegstra, 1986, Schreier *et al.*, 1985, Smeekens *et al.*, 1987, van den Broeck *et al.*, 1985, Wasmann *et al.*, 1986).

Euglena gracilis is a unicellular alga and the chloroplasts of *Euglena* have envelopes consisting of triple membranes (Gibbs, 1970, Lefort-Tran, 1980), in contrast to the double membrane of higher plants. The nature and origin of the outermost membrane of the chloroplasts is not clear. The membrane has been

suggested to be derived from ER or from the plasma membrane of the endosymbiont in the secondary endosymbiotic events. Another unusual feature of chloroplasts from *Euglena*, which is not observed with the chloroplasts of higher plants, is that two or three thylakoids are appressed along the entire length of the chloroplast. These characteristics in the structure of chloroplasts in *Euglena* may impose various restriction on the process of import of precursor proteins in chloroplasts and translocation of the proteins across the thylakoid membranes.

To date, the structure of a few nucleus-encoded precursor proteins from *Euglena* has been analyzed. The precursors of the small subunit of Rubisco (SSU) (Chan, 1990) and the apoprotein of light-harvesting chlorophyll protein (LHCPII) of photosystem II (PS II) (Rikin and Schwartzbach, 1988) were shown to be synthesized as polyproteins in the cytosol. By contrast, the precursors to porphobilinogen deaminase (PBGD), which is an enzyme functioning in biosynthesis of tetrapyrrole, is synthesized as a monomeric protein (Shashidhara and Smith, 1991). The transit peptides of the precursors of SSU, PBGD and LHCP II of *Euglena* are much longer than those of the corresponding proteins in higher plants (134, 139 and 141 amino acids, respectively), and they have N-terminal hydrophobic domain consisting of about 35 amino acids, which is similar to the signal sequence (Chan, 1990, Sharif, 1989, Kishore *et al.*, 1993)(Table I). No homology has been identified to date between these N-terminal hydrophobic domains and the amino acid sequences of the chloroplast transit peptides of higher plants.

Previously, Shashidhara *et al.* showed that the precursor to *Euglena* PBGD was efficiently translocated across the envelope of *Euglena* chloroplasts and processed to the mature size, while the import was abolished by the removal of the first 30 amino acids from the N-terminus, which compose the signal peptide domain of the presequence (Shashidhara *et al.*, 1992). These results indicate that a signal peptide-like structure is required for translocation of the precursor

Table I. Comparison of the number of amino acid residues of determined nucleus-encoded chloroplast precursor proteins in *Euglena*.

Protein	Number of amino acid residues			Note
	Transit peptide	Mature	Precursor	
[Stromal protein]				
Small subunit of Rubisco	134	1070	1274	Polyprotein
Porphobilinogen deaminase	139	341	480	Monomeric protein
[Thylakoid membrane protein]				
Apoprotein of LHCII	141	nd	nd	Polyprotein

across the outermost membrane of the envelope in *Euglena* chloroplasts (Shashidhara *et al.*, 1992). The precursors of nucleus-encoded proteins of *Euglena* have N-terminal hydrophobic domains in their transit peptides generally, which is suggested to be required for translocation of the proteins across the envelopes of chloroplasts consisting of triple membranes.

In this thesis, the author identified the precursor of the nuclear-encoded 30 kDa protein (preOEC30) associated with PS II in *Euglena* chloroplast (Chapter I), and the structure of this precursor protein was analyzed and characterized (Chapter II). In Chapter III, from the *in vitro* import experiments of *Euglena* preOEC30 to chloroplasts in heterologous systems, the author characterized the transit peptide of preOEC30 which includes two hydrophobic domains.

CHAPTER I

IDENTIFICATION OF preOEC30 IN *EUGLENA*

Euglena has been shown to have specific features in the structure of precursors of the nuclear-encoded proteins such as SSU, LHCP I (Houlné and Schantz, 1988), LHCP II and PBGD.

In this chapter, the author identified preOEC30 in *Euglena*, in order to investigate whether the type of precursor synthesized in the cytosol in *Euglena* is related to the final state of the mature protein. The OEC30 corresponds to the so-called OEC33 in higher plants (Mizobuchi and Yamamoto, 1989), which is one of the extrinsic proteins at the lumenal side of thylakoid and required for oxygen evolution in chloroplast (Yamamoto *et al.,* 1981, Yamamoto and Nishimura, 1983, Yamamoto, 1988). The mature OEC30 exists as a monomer and constitutes the multimeric PS II complex with other extrinsic and intrinsic membrane proteins (Yamamoto, 1989)(Fig. 2).

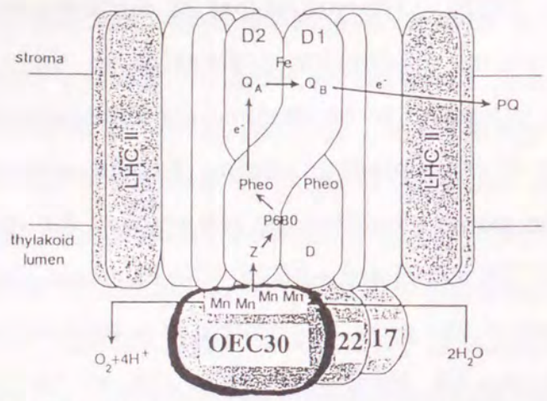

Fig. 2. PS II reaction center. PS II is a multi-subunit complex consisting of several proteins, photosynthetic pigments and other components. Extrinsic proteins are encoded by nucler DNA (shaded).

Materials and Methods

Materials.

The cells of *Euglena gracilis* Z were grown either in the dark or light at 25 °C in a heterotrophic medium with stirring (Hiramatsu *et al.,* 1991). In the light condition, the cells were illuminated with fluorescent lamps with intensity of 330 μE m^{-2} s^{-1}. Messenger-activated paper was obtained from Takara biomedicals. Nuclease-treated rabbit reticulocyte lysate was from Amersham International plc, [^{35}S]-Methionine (22.2 MBq ml^{-1}, specific activity 37 MBq $nmol^{-1}$) and [^{35}S]-Sulfate (0.74 MBq ml^{-1}, specific activity 37 MBq $nmol^{-1}$) were from ICN Biomedicals Inc. Protein-A Sepharose was from Sigma chemical company.

RNA isolation and *in vitro* translation.

Total RNA was extracted according to the protocol of Schuler and Zielinski (Schuler and Zielinski, 1989) from the cells grown in the dark for a week or grown in the light for 3 to 4 days. As a control, RNA was also isolated from leaves of pea grown in the light for a week at 25°C. Poly(A)$^+$ mRNA was obtained from the total RNA with a messenger-activated paper. The mRNA was translated in vitro with a nuclease-treated rabbit reticulocyte lysate at 30°C for 1h in the presence of [^{35}S]-methionine. The reaction was terminated by the addition of cold methionine solution (10 mM).

Growth and labeling conditions of cells and *in vivo* pulse-chase.

The *Euglena* cells, grown in a low sulfate medium (Monroy *et al.,* 1987) for 4 to 5 days in the dark, were aseptically collected by centrifugation, resuspended in a resting medium (pH 6.8) (Horrum and Schwartzbach, 1980) and stood for 3 days in the dark. Development of chloroplast was initiated by exposing the cells to the light with the intensity described above, and after 23 h of

illumination, the cells were pulse-labelled with [^{35}S]-sulfate. A pulse-chase experiment was carried out as described by Rikin and Schwartzbach (Rikin and Schwartzbach, 1988) with partial modification. Chase was initiated by the addition of $MgSO_4$ to a final concentration of 0.1 M. The cells were harvested by centrifugation at 2,000 x *g* for 2 min, and the proteins were solubilized by suspending the pellet in a solution containing 60 mM Tris-HCl pH 8.6 and 1.0% (w/v) SDS followed by boiling for 2 min. Where indicated, 3.0% (w/v) SDS was used for the solubilization instead of 1.0% SDS.

Immunoprecipitation.

Immunoprecipitation was done according to the protocol of Perbal (Perbal, 1988). The *in vitro* translation products of mRNA and the solubilized protein fraction in the pulse-chase experiment were mixed with 940 ml of NET/NP-40 buffer (0.5% Nonidet P-40/0.15 M NaCl/5 mM EDTA/0.2% BSA/0.05 M Tris-HCl, pH 7.5). Samples were preadsorbed with 30 ml of protein-A Sepharose for 30 min at 4°C and then centrifuged for 4 min at 10,000 x *g*. The supernatant was shaken for 15 h at 4°C in the presence of each of polyclonal antibodies against spinach OEC33, against LHC II. After incubation, 30 ml of protein-A Sepharose was added, and the mixture was incubated for 30 min at 4°C with shaking. The pellet was washed five times with NET/NP-40 buffer containing 0.5 M NaCl and twice with NET/NP-40 buffer containing 0.25 M NaCl and twice with buffer containing 0.15 M NaCl without detergent. The final pellet was eluted with sample buffer (3% (w/v) SDS, 10% (w/v) glycerol, 0.1 M DTT, 0.004% bromophenol blue, 65 mM Tris-HCl, pH 6.8) for 5 min at 95°C. The immunoprecipitated proteins were separated by a 12.5% SDS-PAGE according to Laemmli (Laemmli 1970). Fluorography was carried out according to Laskey and Mills (Laskey and Mills 1975). To detect the mature OEC30 in *Euglena* or the OEC33 in pea, western blotting was performed as described (Yamamoto, 1988).

Results

Identification of preOEC30 by in vitro translation.

Euglena preOEC30 detected by immunoprecipitation of translation products of mRNA with a specific antibody had an apparent molecular weight of 45,000 in SDS-PAGE (Fig. 3A). There was no other protein immunoprecipitated and detected at the higher or lower molecular weight range of the gel. Under the same condition, the precursor of the OEC33 in pea which is equivalent to the OEC30 in *Euglena* was shown to have a molecular weight of 38,000. The molecular weights of the precursor and the mature forms of the OEC33 in pea were determined precisely from the nucleotide sequences of cDNA (Wales *et al.*, 1989). A considerable homology exists in the sequences of amino acid for the OEC33 and their precursors from several species of higher plants (Ko *et al.* 1990, Tyagi *et al.*, 1987). It is suggested from the mobility of protein in SDS-PAGE, that preOEC30 in *Euglena* has a longer transit peptide than the precursors of the corresponding proteins from higher plants. Precursors of the apoproteins of LHC II of *Euglena* were also identified with the same procedure (Fig. 3B). Extremely large precursors of the apoprotein of LHC II were detected at the range of molecular weight from 80,000 to 200,000 as reported by Rikin and Schwartzbach (Rikin and Schwartzbach, 1988). Presence of an extremely subunit of Rubisco (data not shown).

Identification of preOEC30 by pulse-chase labelling.

Euglena preOEC30 was identified by another way, i.e. a pulse-chase experiment and the following immunoprecipitation (Fig. 4). After labelling the cells with [^{35}S]-sulfate for 1 min, the precursor having a molecular weight of 45,000 was detected in the gel of SDS-PAGE. Accumulation of the 45 kDa precursor reached the maximum level after 5 min of labelling, and a small amount of the mature OEC30 appeared in the same time. In the following chase

precursor reached the maximum level after 5 min of labelling, and a small amount of the mature OEC30 appeared in the same time. In the following chase period, the mature protein accumulated with time and the amount of the precursor decreased in parallel. The absence of an extremely large precursor for OEC30 suggests that there is a mechanism which determines the form of the precursor depending on the mode of assembly of the protein. With relation to the processing, we estimated the half-time of accumulation of mature protein in the pulse-chase experiment (Fig. 4). PreOEC30 was slowly processed to the

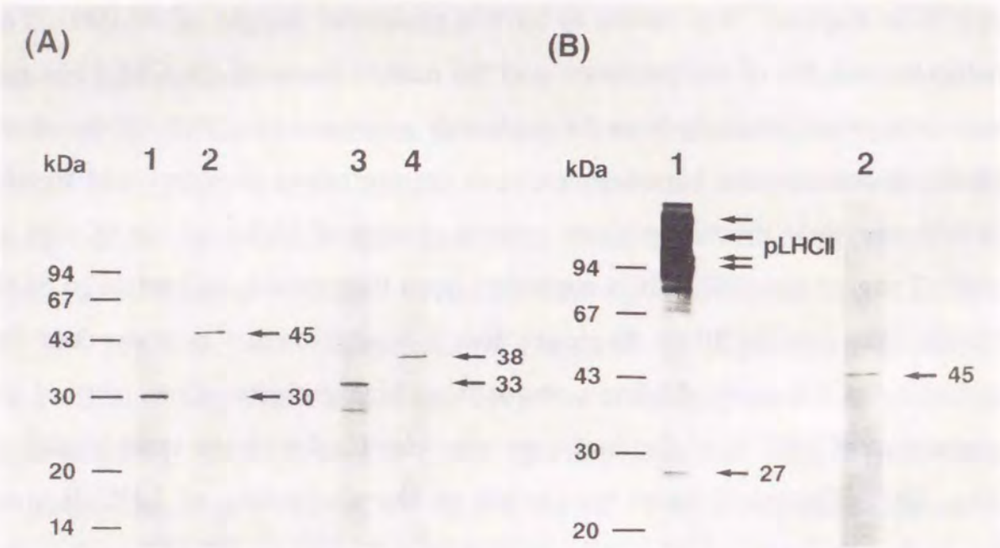

Fig. 3. Detection of the precursor and mature form of the OEC33 from *Euglena* and pea, and precursor of the apoprotein of LHC II from *Euglena*. A. Lane 1, OEC30 from *Euglena* ; lane 2, the precursor of the OEC30 from *Euglena* ; lane 3, OEC33 of pea ; lane 4, the precursor of the OEC33 from pea. B. Lane 1, the precursor of the apoprotein of LHC II from *Euglena* ; lane 2, the precursor of the OEC30 from *Euglena*. For identification of the precursors, immuno-precipitation of *in vitro* translation products of poly(A)+RNA with a specific antibody, SDS-PAGE and fluorography were carried out (lane 2 and 4 in A and lane 1 and 2 in B). The mature forms of the proteins were detected by SDS-PAGE and western-blotting analysis (lanes 1 and 3 in A). Marker proteins of the molecular weight are phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase from bovine erythrocyte (30 000), soybean trypsin inhibitor (20,100) and a-lactalbumin (14,400). The bands of the proteins detected are shown by arrows.

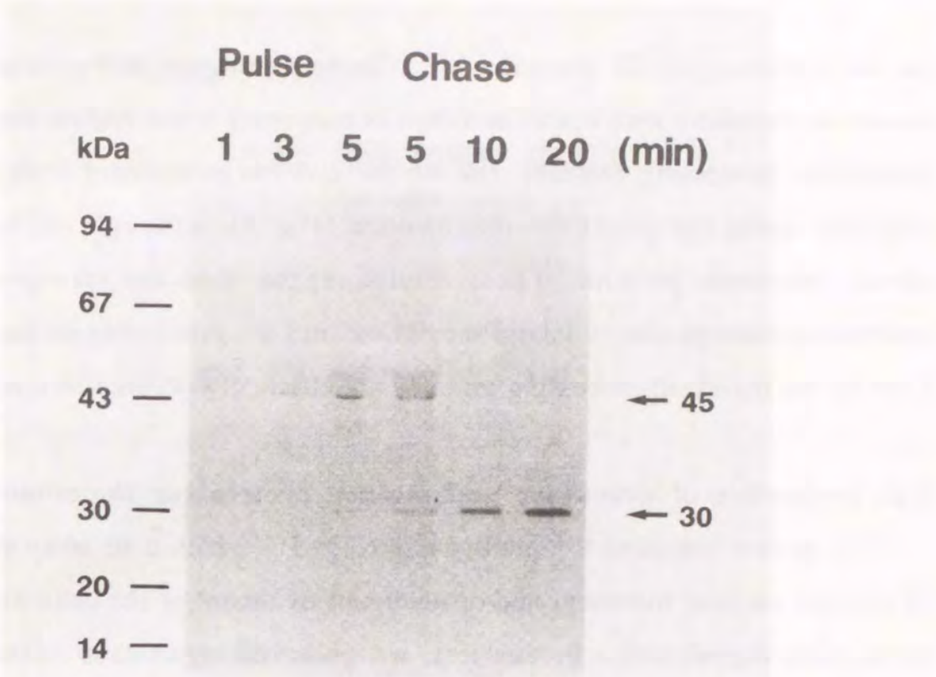

Fig. 4. *In vitro* pulse-chase experiment showing the processing of the precursor of the OEC30 to the mature form in *Euglena* cells. The cells grown in a low sulfate medium for a week and then illuminated for 23 h. The cells were pulse-labelled in the following light period with [^{35}S] sulfate for 1, 3, and 5 min, and chased for 5, 10 and 20 min. The bands of the precursor and mature proteins are shown by arrows.

mature form, and the half-time was about 10 min under the present condition. Under a similar condition, the half-time for maturation of LHC II was shown to be about 20 min, whereas mitochondrial enzyme fumarase was quickly processed within 10 min of pulse-labelling period (Rikin and Schwartzbach, 1988). Although the half life-time for the processing of the precursor may be dependent on the growth condition of the cells, the OEC30 is apparently a member of the slowly processed proteins in *Euglena*.

In the processing experiment of OEC33 *in vitro* using the precursor and the isolated intact chloroplasts, an the processing intermediate of OEC33 was detected (James *et al.*, 1989, Watanabe, 1987). The intermediate results from

partial processing of the precursor by a stromal protease, and is transported across the thylakoid membrane, and then is processed to the mature form by a thylakoidal processing protease. In the pulse-chase experiment with *Euglena* cells, we could not detect the intermediate (Fig. 4), although preOEC30 is slowly processed protein. These results suggest that the transporting of intermediate across the thylakoid membrane and the processing to the mature form by the thylakoid processing protease are efficiently occured *in vivo*.

The properties of precursor and mature protein for the solubility.

The author carried out 'solubility test' (Fig. 5), which is to assay solubility of proteins on heat treatment and/or detergent treatment of the cells during the pulse-chase experiment. In this test, we observed significant difference in solubility between the mature and precursor forms of the OEC30. The mature protein was easily released from the thylakoid membrane by heat treatment at 100°C for 2 min as was reported previously with chloroplast (Yamamoto and Nishimura, 1983), and detected by immunoprecipitation. On the other hand, the precursor was not released from the membrane fraction by the simple heat treatment and further treatment with SDS (1-3%) was required for the solubilization (Fig. 5). The author estimate that the presequence of preOEC30 is rich in hydrophobic amino acid residues and/or that the preOEC30 is once translocated and stay in the membrane system other than chloroplast membrane before the final assembly.

Discussion

The author found here that *Euglena* preOEC30 is not synthesized as a polyprotein. However, it probably has a longer transit peptide compared with the corresponding precursor of OEC33 from higher plants. Although translated

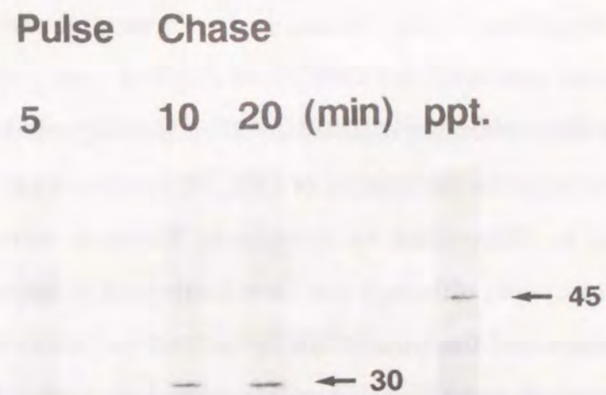

Fig. 5. Solubility test of the precursor and the mature protein in the pulse-chase experiment. The *Euglena* cells were pulse-labelled for 5 min and chased for 10 and 20 min. After the pulse and each chase period, the cells were heat-treated at 100 °C for 2 min. The mature form was easily solubilized (released from the thylakoid membrane) by the heat treatment, whereas the precursor was not. In the far right lane (designated as ppt), the cells pulse-labelled for 5 min were heat-treated and then centrifuged. The pellet obtained was treated with 3% SDS and boiled for 2 min. Under these conditions, the 45 kDa precursor was released from the membrane into a soluble fraction and detected by immunoprecipitation.

as a non-polyprotein type precursor, the precursor of OEC30 seems to be processed in a way characteristic to *Euglena*. The absence of an extremely large precursor for OEC30 suggests that there is a mechanism which determines the form of the precursor depending on the mode of assembly of the protein. Synthesis of the precursor of the nuclear-encoded proteins as polyproteins, as was observed with SSU and LHC, may be beneficial to *Euglena* cells, when the transport pathway of the precursor proteins from cytoplasm to chloroplast is complex and the assembly of the protein is limited at the transport process.

the pulse-chase experiment, the presence of a large precursor and the slow processing observed here with the OEC30 of *Euglena* are probably related to the complexity in the processing of the protein in this organism. There may be several limiting steps in the maturation of OEC30; synthesis and transport of the precursor protein to chloroplast in cytoplasm, transport across the envelope membrane of chloroplast, although the transporting of intermediate across the thylakoid membrane and the processing to the mature form by the thylakoid processing protease seem to be efficiently occured *in vivo*. Structure of the precursor of the nuclear-encoded protein in this organism may be closely related not only to the characteristics of protein transport, but also to the feature of assembly of the protein.

Summary

Polyprotein-type precursors have been reported for the nuclear-encoded proteins such as SSU, LHCP I and LHCP II in *Euglena*. The author describe here that the precursor of the extrinsic 30 kDa protein of photosystem II (preOEC30) encoded by nuclear DNA is not a polyprotein. The precursor was identified as a 45 kDa protein by immunoprecipitation of *in vitro* translation products of mRNA and by a pulse-chase experiment. It is probable that the structure of the precursor of the nuclear-encoded protein in *Euglena* chloropalst is closely related to the feature of assembly, as well as of transport, of the protein in chloroplast.

CHAPTER II

ANALYSIS AND CHARACTERIZATION OF STRUCTURE OF preOEC30

As described in Chapter I, preOEC30 has a longer transit peptide compared with the corresponding precursor of the OEC33 from higher plants, and the processing of preOEC30 occurs slowly. There may be several limiting steps in the maturation of the OEC30 : biosynthesis and transport of the precursor protein to chloroplast in cytoplasm, transport across the envelope membrane of chloroplast. Schwartzbach and Osafune *et al.* showed that the precursor to *Euglena* LHCP II is synthesized as a polyprotein on the membrane-bound polysomes, and transported to ER and Golgi apparatus prior to chloroplast localization (Kishore *et al.*, 1993, Osafune *et al.*, 1991, Schiff *et al.*, 1991).

First, the author investigated whether *Euglena* preOEC30 is synthesized on free or membrane-bound polysomes in cytoplasm. As the precursor of *Euglena* OEC30 seems to be synthesized on the free ribosomes in the cytoplasm, one of the rate-limiting steps in the maturation of the protein should be the translation of the precursor across the envelope. The chloroplast envelopes of *Euglena* consists of three membranes, in contrast to the double membrane of higher plants (Gibbs, 1970, Lefort-tran, 1980). The outermost membrane of the envelope of *Euglena* has been suggested to be ER-like although no ribosomes are present on the surface of this membrane (Gibbs, 1981). The structure of the chloroplasts of *Euglena* may impose various restriction on the process of import of precursor proteins in chloroplasts. The author observed the envelope membranes of the chloroplasts by electron microscopy, in particular, the contact site which have been suggested to be the sites of translocation of

precursor proteins (Pain *et al.*, 1988), because it is not known with *Euglena* how the contact site is formed in the envelope.

To clarify the possible relationship between the structure of precursors and the pathway for their transport to chloroplasts in *Euglena*, the author isolated a cDNA for OEC30, and characterized the structure of the precursor protein.

Material and Methods

Growth of *Euglena*.

The cells of *Euglena gracilis* Z were grown either in the dark or light at 25°C in a heterotrophic medium with stirring (Hiyamatsu, 1991). In the light condition, the cells were illuminated with fluorescent lamps with intensity of 330 μE m^{-2} s^{-1}.

Preparation of polysomes.

Free and membrane-bound polysomes were isolated according to Larkins *et al.* (Larkins, 1975, Larkins *et al.*, 1976). The *Euglena* cells, grown in heterotrophic medium at 25°C for 5 or 6 days in the dark, were transfered to the light for 24 or 36 h. After illumination, the cells were harvested, immediately frozen into liqid nitrogen. The frozen *Euglena* cells were milled in an ice-cold mortar. The flour was then suspended in buffer A (0.2 M Tris-HCl, 0.2 M sucrose, 60 mM KCl, 50 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and 5 mM ß-mercaptoethanol, pH 8.5), broken by the homogenizer. The homogenate was centrifuged at 500 x *g* for 10 min. The supernatant was centrifuged at 1,300 x *g* for 10 min, and then at 37,000 x *g* for 10 min to separate free and membrane-bound polysomes. The supernatant containing free polysomes was layered over 4 ml of 60% (w/v) sucrose in buffer B (40 mM Tris-HCl, 20 mM KCl, and 10 mM $MgCl_2$, pH 8.5), while the pellet was

suspended in buffer A containing 1% (v/v) Triton X-100 and 0.5% DOC (sodium deoxycholate) and centrifuged at 35,000 x *g* for 10 min. The supernatant was layered over 4 ml of 60% (w/v) sucrose in buffer B. Both free and membrane-bound ribosomes were pelleted by centrifugation for 2 h at 23,000 x *g* in the 65 rotor of a Hitachi ultracentrifuge. Polysome pellets were washed in buffer B, and resuspended in the polysome solution (40 mM Tris-HCl, 70 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.25 M sucrose, pH 7.6).

***In vitro* translation and immunoprecipitation.**

Translation assays were carried out by the methods described in Chapter I. Instead of poly(A)$^+$mRNA, both polysomes were added to each translation mixture. Immunoprecipitation of labelled proteins was performed using anti OEC33 antibodies as described in Chapter I.

Electron microscopy.

The *Euglena* cells, grown for 4 to 5 days in the dark, were aseptically collected by centrifugation, resuspended in a resting medium (pH 6.8) (Horrum and Schwartzbach, 1980) and stood for 3 days in the dark. Development of chloroplast was initiated by exposing the cells to the light with the intensity described above, and after 8 h of illumination, the cells were harvested. Gultaraldehyde was added to the culture at a final concentration of 2.0% (v/v) and incubated at 4°C for 2 h. The cells were centrifuged at 250 x *g* for 3 min at 4°C then resuspended in 0.05 M phosphate buffer, pH 7.0. The resuspend cells were washed 3-4 times with the same buffer. The pellet of the cells was suspended in 1.0% osmium tetroxide in water then incubated at 4°C for 2 h. The incubated cells were washed 3-4 times with the distilled water, and embedded in 2% agar and dehydrated in a 50 to 90% ethanol series followed by propyleneoxyside. The material was embedded in Epok 812 resin, sectioned

and stained with 2.0% uranyl acetate (Ehara *et al.*, 1984), then with lead citrate. Sections were examined with a Hitachi H-150 electron microscope.

Cloning of cDNA for OEC30.

Cloning of a cDNA for OEC30 was carried out by the polymerase chain reaction (PCR) technique (Saiki *et al.*, 1988). First-strand cDNA was synthesized from purified polyadenylated [poly $(A)^+$]mRNA from photoheterotrophically grown *Euglena* cells using oligo(dT) primers and reverse transcriptase from Moloney leukemia virus. A pair of specific oligomers was added to the single-stranded cDNA, and PCR was carried out with the cDNA population as template. The mixed oligonucleotide primers used for amplification were designed by reference to the consensus sequence of amino acids in the C-terminal side of OEC33 from pea and *Chlamydomonas*. The first synthetic oligonucleotide primer (oligonucleotide 1), consisting of 23 nucleotides, was complementary to the sequence that encodes the heptapeptide Thr-Gly-Tyr-Asp-Asn-Ala-Val, and also included two nucleotides from the codon for the eighth amino acid, alanine. The second oligonucleotide (oligonucleotide 2) was a 17mer corresponding to the amino acid sequence of the pentapeptide Gln-Pro-Ser-Asp-Thr, plus two additional nucleotides from the codon for the sixth amino acid, aspartic acid.

Oligonucleotide 1 (23- mer)

```
5' - ACG GGG TAC GAC AAC GCG GTG GC -3'
     A   A   T   T   T   A   A
     T   T                   T   T
     C   C                   C   C
```


Oligonucleotide 2 (17- mer)

```
3' - GTC GGG AGG CTG TGG CT -5'
     T   A   A   A   A
         T   T       T
         C   C       C

3' -         TCG             - 5'
             A
```

After PCR, fragments of expected length were isolated by gel electrophoresis (1.5% agarose) and subcloned into plasmid vector pBluescript II (StrataGene, U.S.A.). One of them included an open reading frame that showed considerable homology to the amino acid sequences from OEC33 of spinach (Tyagi *et al.*, 1987), pea (Wales *et al.*, 1989) and *Anacystis nidulans* (Kuwabara *et al.*, 1987). Therefore, we concluded that the plasmid had an insert cDNA that encoded part of OEC30 of *Euglena*. We used this DNA fragment as a probe for further screening of clones from a cDNA library. The PCR-extended products were labeled with [α-^{32}P]dATP by PCR, as described by Hara-Nishimura *et al.* (Hara-Nishimura *et al.*, 1990). The library was constructed by the vector-primer method with pBluescript II, as described by Mori *et al.* (Mori *et al.*, 1991). After repeating the screening three times, we obtained 10 positive clones. DNA was isolated from the recombinant plasmid clones by the alkaline lysis method. After double digestion with *Sac* I and *Kpn* I, the lengths of inserts were analyzed by electrophoresis on a 1.2% agarose gel. The nucleotide sequence of the largest clone was determined by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1980) with T7 DNA polymerase (Pharmacia, Sweden).

Preparation of genomic DNA and Southern blotting.

Preparation of genomic DNA and Southern blotting were carried out according to the protocol in reference Sambrook *et al.* (Sambrook *et al.*, 1989). *Euglena* cells grown under white fluorescent lamps (330 μE m^{-2} s^{-1}) and at the

exponential stage of growth were collected by centrifugation at 500 x *g* for 3 min. The cells (about 8 g wet weight) were extracted successively with SDS/phenol, phenol/chloroform (twice) and chloroform/isoamyl alcohol. Nucleic acids were precipitated by the addition of one volume of isopropanol, suspended in TE buffer and precipitated by the addition of two volumes of ethanol. The precipitate was then suspended in TE buffer and incubated at 4°C overnight after the addition of 0.25 volumes of 8 M urea and 0.32 volumes of 10 M LiCl. Then after centrifugation at 4°C, the supernatant was mixed with two volumes of ethanol and genomic DNA was obtained as the precipitate. The genomic DNA was digested with *Hind* III and *Kpn* I and subjected to electrophoresis on a 0.8% agarose gel. The DNA was depurinated by soaking the gel in 0.25 M HCl for 10 min and was capillary-blotted onto a Hybond N+ membrane (Amersham, Japan) over the course of 4 h in the presence of 0.4 M NaOH. The membrane was washed with 2 x SSPE solution and prehybridized for 2 h at 42°C in 20 ml of 6 x SSPE that contained 5% Denhardt's solution, 0.5% SDS and 50% formamide. The hybridization buffer was then replaced by the same buffer solution that contained about 25 ng of cDNA fragment that had been labeled with [^{32}P]-dCTP by the random-primer DNA-labeling method, and the membrane was incubated overnight in this solution at 42°C. The membrane was washed then twice with 2 x SSPE that contained 0.1% SDS at 65°C and then with 0.2 x SSPE that contained 0.1% SDS at 65°C. The membrane was then exposed to X-ray film (Fuji, Japan) under an intensifying screen at -70°C for 24 h.

Preparation of total RNA and Northern analysis.

Total RNA was obtained as a precipitate after treatment with LiCl, as described above. The precipitate was suspended in TE buffer and poly (A)+RNA was isolated by affinity chromatography on a column of oligo(dT) cellulose. The poly (A)+RNA was denatured by treatment with formamide and

subjected to electrophoresis on a 1% agarose gel that contained 18% (v/v) formaldehyde. The RNA was vacuum-blotted onto a Hybond-N+ membrane in 0.5 M ammonium acetate and cross-linked by exposure to UV light. Northern hybridization was carried out as described in Southern blotting. Hydropathy analysis of the amino acid sequence was carried out by the method of Kyte and Doolittle (Kyte and Doolittle, 1982) with a window size of 11 amino acids.

Results

Biosynthesis of preOEC30 in cytoplasm.

The author separated membrane-bound and free polysomes from *Euglena* cells, and examined the translation products with immunoprecipitation using antiOEC33 antibodies, then analyzed by SDS-PAGE and fluorography (Fig. 6A). As the immunoprecipitate of *Euglena* preOEC30 was detected mainly in the translation products synthesized in the presence of free polysomes, it was suggested that the precursor of *Euglena* OEC30 was produced on the free polysomes. The size of the precursor of *Euglena* OEC30, which is in fact present *in vivo*, detected by a pulse-labelling experiment was corresponds to the size of the precursor detected by immunoprecipitation of *in vitro* translation product (Fig. 6B). These results indicate that the precursor of *Euglena* OEC30 is synthesized on the free ribosomes in the cytoplasm and transported post-translationally to chloroplasts, although the preOEC30 has a longer transit peptide and the processing was more slowly.

Structure of envelope.

Although *Euglena* preOEC30 is seems to be synthesized on the free ribosomes in the cytoplasm, preOEC30 has a longer transit peptide compared with the corresponding precursor of OEC33 from higher plants, and that the

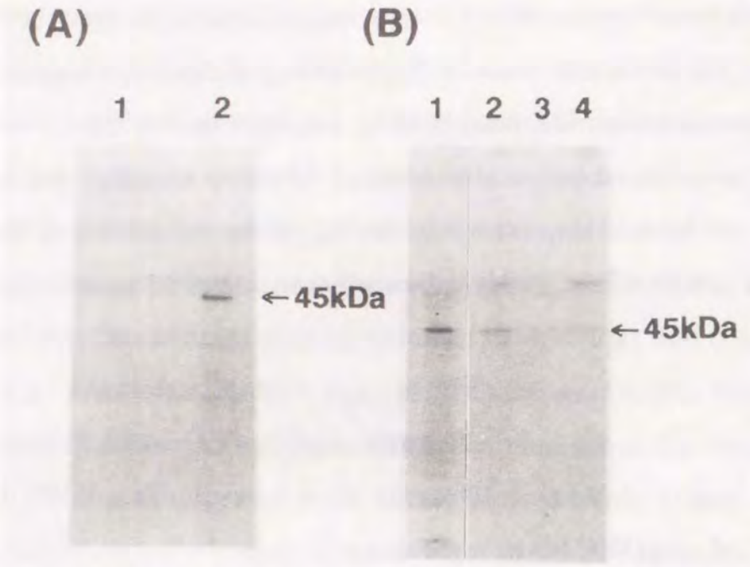

Fig. 6. Immunoprecipitated polypeptides synthesized on membrane-bound and free polysomes, and molecular masses of the *in vitro* and *in vivo* translation products. A. Membrane-bound and free polysomes were translated in a nuclease-treated rabbit reticulocyte lysate in presence of [^{35}S]-methionine. The products were immunoprecipitated with anti-OEC33 antibodies corresponding to membrane-bound polysomes (lane 1) and to free polysomes (lane 2). B. Lane 1 and 3, OEC33-immuno-precipitation of *in vitro* translation products of poly(A)$^+$RNA with a specific antibody ; lane 2 and 4, OEC33-immuno-precipitation of *in vivo* translation products obtained by pulse-labeling *Euglena* cells for 5 min.

processing of preOEC30 occurs slowly. One of the rate-limiting steps in the maturation of the protein should be the translation of the precursor across the envelope. The author examined the characteristics of the contact site by electron microscopy. It was not easy to differentiate the envelope and the thylakoid membrane in fully developed chloroplast, because the thylakoid membranes extend over the length of chloroplast and grana stacking of the thylakoid usually detected in chloroplast of higher plants dose not exist in *Euglena* chloroplast. For this reason, the author used the chloroplasts from *Euglena* cells, which were grown in the dark and illuminated for a short period (8 h) to develope chloroplast partially. Electron micrographs showed that the

envelope membranes of *Euglena* chloroplasts consisted of triple membranes (Fig. 7-9). The outermost perichloroplastic membrane and the intermediate one, or the intermediate membrane and the most internal one were found to contact in places each other (Fig. 7,8). The contact sites, which were formed by association of all the three membranes, were occasionally observed (Fig. 8,9). These results suggest that the outermost membrane of the chloroplast envelope is a significant barrier for the translocation of the precursors, or that the small amount of the contact sites formed by all the three membranes is a rate-limiting step for the process of import of precursor proteis in chloroplasts.

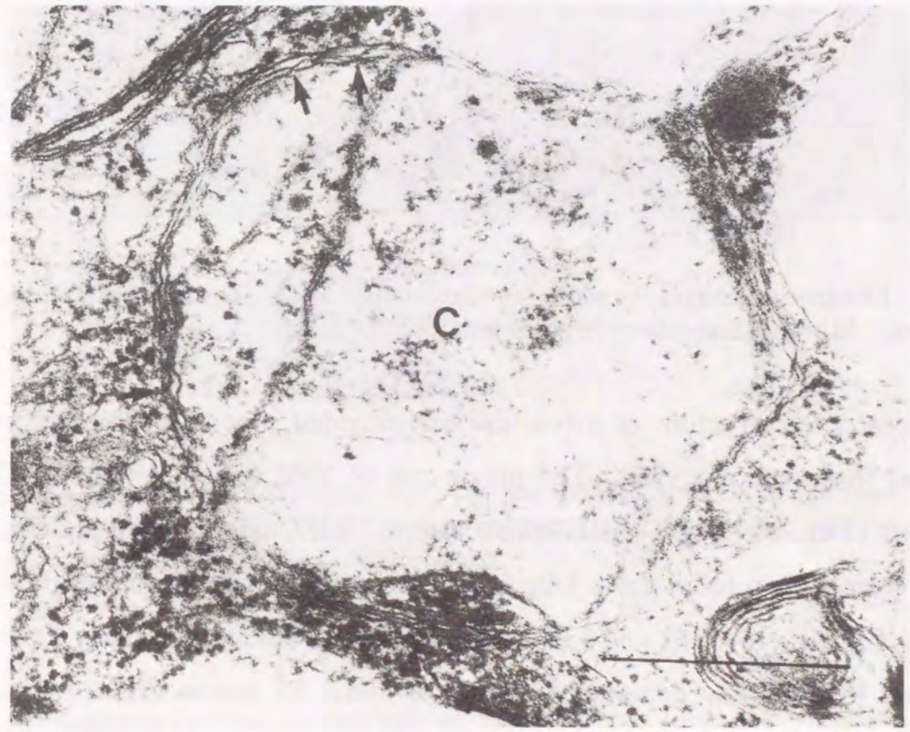

Fig. 7. Electron micrographs of *Euglena* cells grown in the dark and illuminated for 8 h showing the envelope membranes. The envelope consists of three layers of membrane. The outermost perichloroplastic membrane and the intermediate one, or the intermediate membrane and the most internal one contact in places each other (shown by the arrows). The chloroplast observed here is at the early stage of development. The bar corresponds to 1 μm. C, chloroplast.

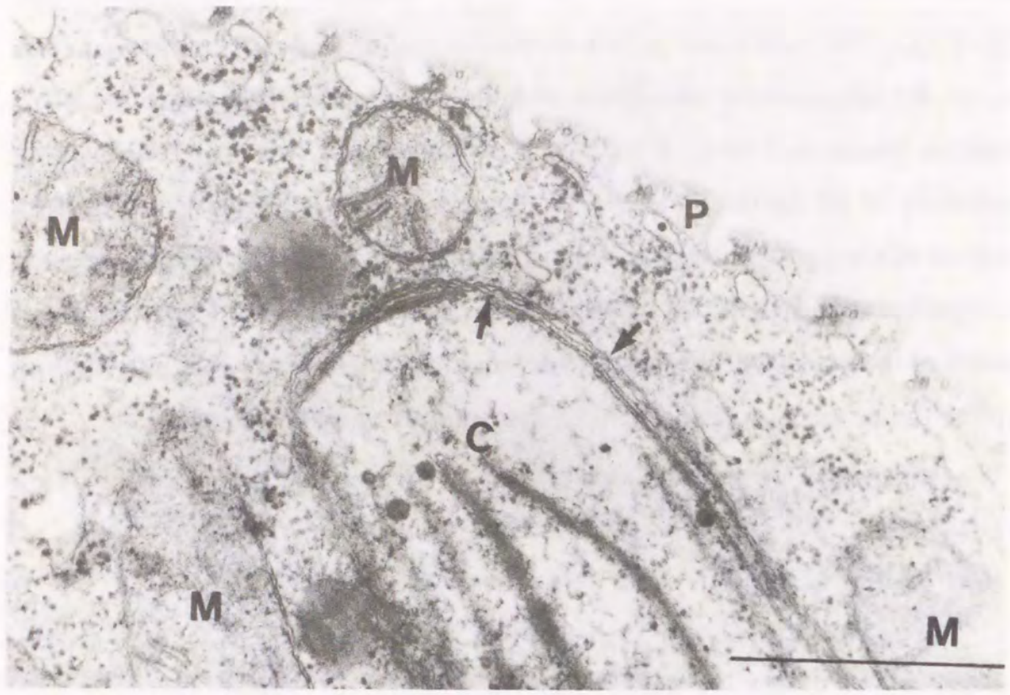

Fig. 8. Electron micrographs showing the contact sites. The bar corresponds to 1 μm. M, mitochondria ; P, pellicle.

To date, the structure of a few nucleus-encoded precursor proteins from *Euglena* has been analyzed. The precursors of SSU (Chan, 1990), LHCPII (Kishore *et al.*, 1993) and PBGD (Sharif *et al.*, 1989, Shashidhara and Smith, 1991) were shown to be much longer than those of the corresponding proteins in higher plants (134, 141 and 139 amino acids, respectively), and they have N-terminal hydrophobic domain consisting of about 35 amino acids, which is similar to the signal sequence, and no homology has been identified to date in the amino acid sequences of the envelope transfer signals of higher plants. The precursors of nucleus-encoded proteins of *Euglena* have N-terminal hydrophobic domains in their transit peptides generally, which is suggested to be required for translocation of the proteins across the envelopes of chloroplasts consisting of triple membranes (Shashidhara *et al.*, 1992).

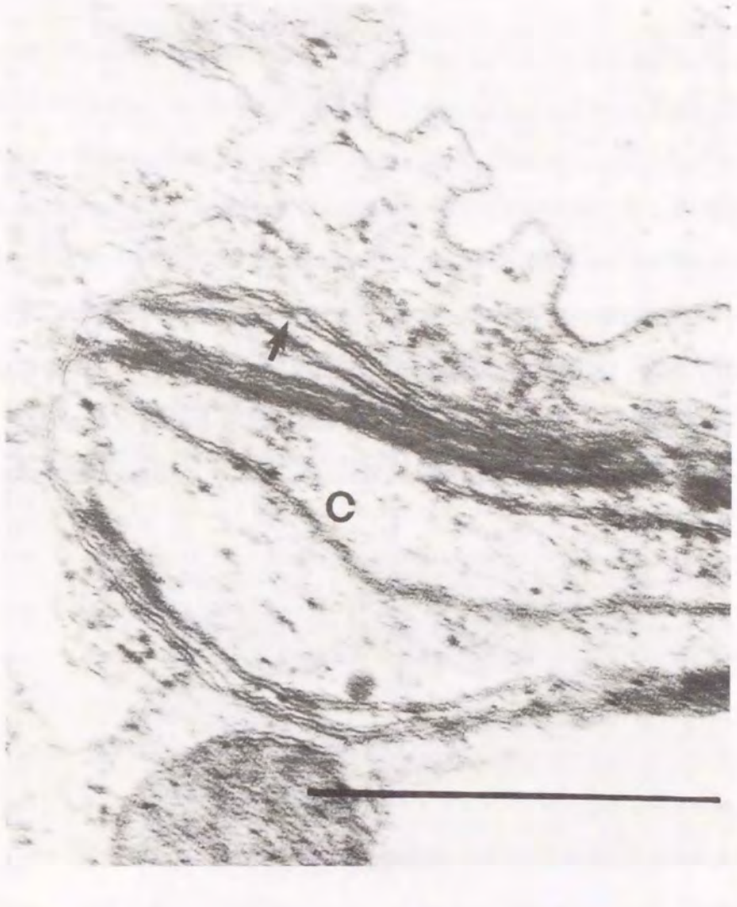

Fig. 9. Electron micrographs showing the contact site. The contact site is formed by association of the three membranes (shown by the arrows). The bar corresponds to 1 μm. C, chloroplast.

Characteristics of the precursor to OEC30.

The author isolated a cDNA for OEC30 to clarify the possible relationship between the structure of the presequence of the precursor and the pathway for their transport to chloroplasts in *Euglena*. The nucleotide sequence of the cDNA and the deduced amino acid sequence showed that the precursor to OEC30 consists of an N-terminal presequence of 93 amino acids and a mature

```
1    AA GAC GTT CAC GTG CAC CGC AAT TGC GCC GTT TCC GTG GGT GTG ACG      47

48   GTC GGC CTG GCC GCT TCG TCT GTC ACC AAC CTC CAT GTC ACC AGC GCC     95

96   CAG ACT CAG GCG GTC ACC GCT GCC GTC CCT GTC ATG AGC CGC GTT AAC    143
1                                                M   S   R   V   N      5

144  TTG GCT GCT CCT GCC AAC CAC GTT GTT CCA CGC GCT GCT CAA GAG GAG    191
6    L   A   A   P   A   N   H   V   V   P   R   A   A   Q   E   E     21

192  GTT CAA GGT GAA TAC CAG ACT GCC TCC AAC AAC TGG GCA GTT GCT GCC    239
22   V   Q   G   E   Y   Q   T   A   S   N   N   W   A   V   A   A     37

240  ATG GCC TCT GCT GGT GCA GCT GTT GGT GCT GCT GTT CTG GCG ATG CGC    287
38   M   A   S   A   G   A   A   V   G   A   A   V   L   A   M   R     53

288  CGC CGT GCC ACC AAC ACC TAT GAG GCC ATC CGC GAG GAT CCG GAG GCC    335
54   R   R   A   T   N   T   Y   E   A   I   R   E   D   P   E   A     69

336  GTT CTG GCT GGC GCT GGT CGT GCA ATG GGC GCT GCC CTC ATC GGA GCT    383
70   V   L   A   G   A   G   R   A   M   G   A   A   L   I   G   A     85

384  GCT GTT GCT GGC TCT GCC AAC GCC GCG TCC CTC ACT TAC GAC GAA CTT    431
86   A   V   A   G   S   A   N   A   A   S   L   T   Y   D   E   L    101

432  CAG TCC CTG TCC TAC TTG GAG GTG AAG AGT TCT GGC ATT GCT GGT ACG    479
102  Q   S   L   S   Y   L   E   V   K   S   S   G   I   A   G   T    117

480  TGC CCA GTT CTT GCT GAT GGC GTC AGC AGC AAG CTG AGC CTG AAG GCT    527
118  C   P   V   L   A   D   G   V   S   S   K   L   S   L   K   A    133

528  GGG AAG TAT GAG ATC AAC AAC TGG TGC TTG GAG CCC TCC TCT TTC CAG    575
134  G   K   Y   E   I   N   N   W   C   L   E   P   S   S   F   Q    149

576  GTG AAA CTT CCC CCT ACG GAG AAG CAG CAG GTA ACC GAG TTC GAA AGG    623
150  V   K   L   P   P   T   E   K   Q   Q   V   T   E   F   E   R    165

624  ACT AAG CTG ATG ACC CGG TTG ACC TAC ACT TTG GAT GCC ATT AGC GCT    671
166  T   K   L   M   T   R   L   T   Y   T   L   D   A   I   S   A    181

672  GAC CTG AAT GTT GGT GGT GAC GGT TCT TGG ACC ATC CAG GAG AAG GAC    719
182  D   L   N   V   G   G   D   G   S   W   T   I   Q   E   K   D    197

720  GGT CTG GAC TAC GCT GCC ACA ACT GTT CAG CTC GCG GGT GGT GAA CGT    767
198  G   L   D   Y   A   A   T   T   V   Q   L   A   G   G   E   R    213

768  GTG CCC TTC CTG TTC ACC ATC AAG AAC TTG CTG GCA AAG GGT GAT GCT    815
214  V   P   F   L   F   T   I   K   N   L   L   A   K   G   D   A    229

816  GGT CAG TTC CTG GGC CAG TTT GAT GTG CCC TCG TAC CGT GGT GCC ACT    863
230  G   Q   F   L   G   Q   F   D   V   P   S   Y   R   G   A   T    245

864  TTC TTG GAC CCC AAG GGA CGT GGT GGT GCA AGT GGG TAT GAC ACC GCT    911
246  F   L   D   P   K   G   R   G   G   A   S   G   Y   D   T   A    261

912  GTT GCC CTG CCT GCC TCT GGT GAT GAC GAG GAG TAT GCG AAG GAG AAC    959
262  V   A   L   P   A   S   G   D   D   E   E   Y   A   K   E   N    277

960  AGC AAG TCC ACC GCT GCT TCA GTT GGC ACC ATT GCC TTC AAG GTT GCC   1007
278  S   K   S   T   A   A   S   V   G   T   I   A   F   K   V   A    293

1008 AAG GTG AAC GCT GAG ACT GGT GAG ATT GCT GGG GTG TTC GAG AGC ATC   1055
294  K   V   N   A   E   T   G   E   I   A   G   V   F   E   S   I    309

1056 CAG CCC TCT GAC ACC GAT CTT GGT GCC AAG GTT CCC AAG GAC ATC AAG   1103
310  Q   P   S   D   T   D   L   G   A   K   V   P   K   D   I   K    325

1104 ACC AGC GGA GTC TGG TAC GCT CAG ATC TCC CCG TCC AAG TAA ACT CTT   1151
326  T   S   G   V   W   Y   A   Q   I   S   P   S   K   *            339

1152 TCA ACC GTG CAG TTG GTG AGC CAC TTT CTC TGA TCA ATT TCC CTC CTG   1199

1200 TTG TAT AAT TTT GAG GTT AGT CCT CAC CCG TTT CTA TGA TAC AAC AGA   1247

1248 CTT CAC CTG AGA GGA GTT GAT TGG TGT ATA AAA AAA AAA AAA AAA AAA   1295
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Fig. 10. Nucleotide and deduced amino acid sequences of OEC30. The arrowhead indicates the cleavage site of the presequence. The N-terminal sequence of the mature polypeptide, determined by microsequencing of the purified polypeptide, is underlined. The termination codon is shown by an asterisk.

protein of 245 amino acids (Fig. 10). There are several candidates for the initiation codon for the methionine residue at the N-terminus of the precursor to OEC30, namely, codons at positions 1, 38, 52 and 78 of the amino acid sequence. Northern blot analysis with the 180-bp cDNA as probe demonstrated that the mRNA that encodes the precursor is about 1.4 kb in length (Fig. 11). In chapter I, the author showed that the molecular mass of the precursor to OEC30 is about 45 kDa by immunoprecipitation of the product of translation *in vitro* of mRNA from *Euglena* cells. The precursor of pea OEC33, which is smaller than that of OEC30 from *Euglena* and consists of an N-terminal presequence of 81 amino acids and a mature protein of 248 amino

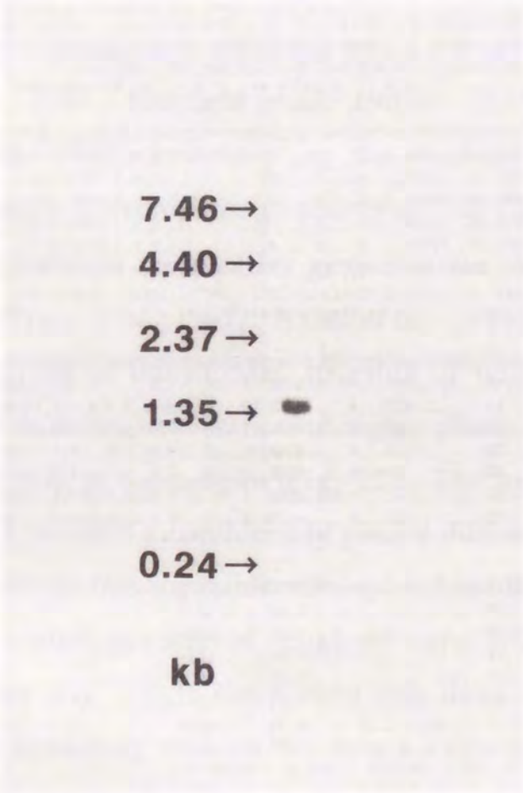

Fig. 11. Northern blot analysis of mRNA with the 180-bp cDNA as probe. The poly(A)+RNA from *Euglena* cells was blotted onto a membrane and allowed to hybridize to the [^{32}P]-labeled probe. The RNA molecular markers are indicated on the right.

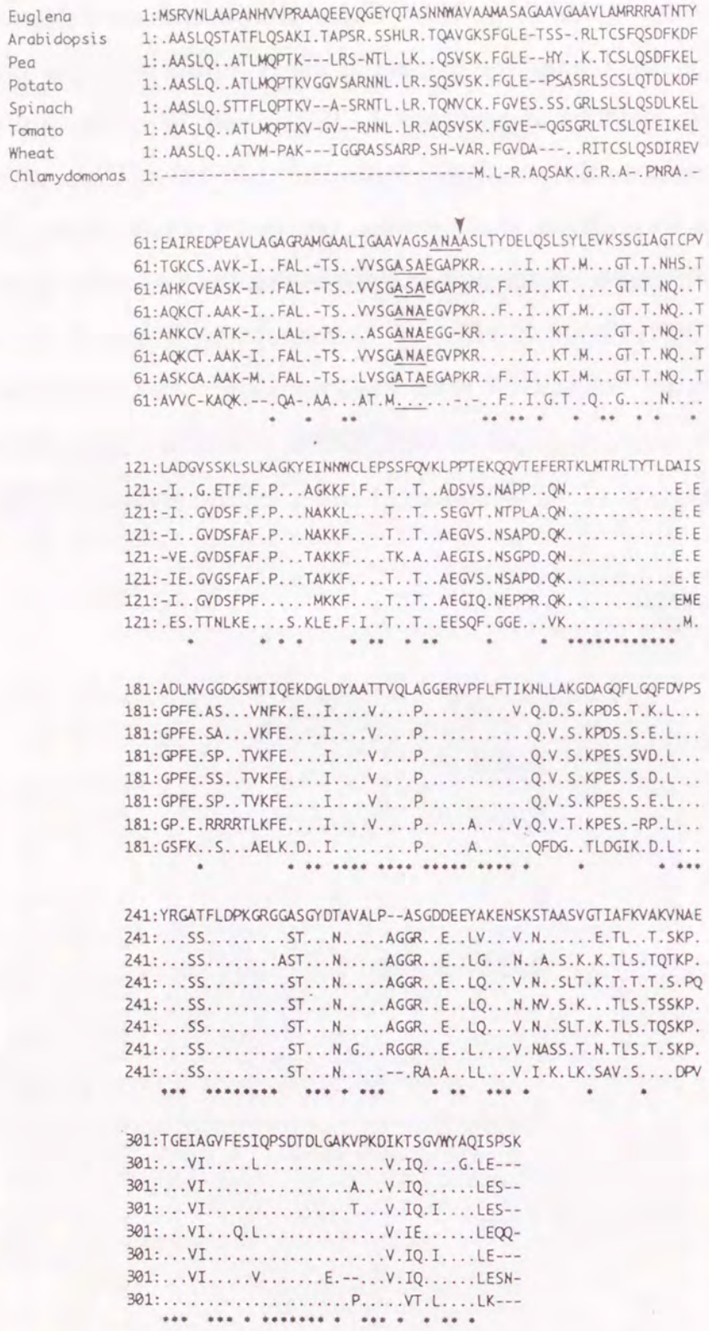

Fig. 12. Comparison of the determined preOEC33 sequences with the sequence deduced from the nucleotide sequence of the *Euglena* OEC30 cDNA clone. Identical residues are shown as *. The thylakoidal processing recognition sites are underlined. The arrowhead indicates the cleavage site of the presequence.

acids (Wales *et al.*, 1989), was identified as a 38-kDa protein by immunoprecipitation/SDS-PAGE analysis. Accordingly, we concluded that translation of the precursor protein starts at the methionine residue designated position 1. The N-terminus of the mature protein was determined by microsequencing of the protein after electro-blotting onto a PVDF membrane. The sequence exactly matched the sequence deduced from cDNA sequencing (Ishikawa and Yamamoto; the details to be presented elsewhere). The alanine residues at positions -1 and -3 positions form a consensus sequence for processing by a specific protease that is present in thylakoids.

Comparison of the deduced amino acid sequences of *Euglena* OEC30 mature protein with the published higher plants and *chlamydomonas* sequences showed homologies of greater than 60% (Fig. 12)(Ko *et al.*, 1990, Tyagi *et al.*, 1987, Wales *et al.*, 1989, Mayfield *et al.*, 1987). The hydropathy profile (Kyte and Doolittle, 1982) generated for the *Euglena* OEC30 sequence indicated a predominantly hydrophilic protein (Fig. 13). The hydropathy plot was considerably similar to the profiles generated for the spinach and pea OEC33 sequences (Tyagi *et al.*, 1987, Wales *et al.*, 1989) (Fig. 13). On the other hand, comparison of the primary structure of transit peptide part of *Euglena* preOEC30 with the presequences of higher plants and *chlamydomonas* indicated the low degree of homologies (Fig. 12). The hydropathy profile of *Euglena* preOEC30 revealed a considerably greater difference between the N-terminal extensions of the transit peptides of *Euglena* and higher plants proteins (Fig. 13). The transit peptide of *Euglena* preOEC30 possessed two hydrophobic domains (Fig. 13), in comparison with those of OEC33 from pea and spinach, which apparently each include only a single hydrophobic domain. The hydrophobic domains identified in the presequences of the latter two species constitute the thylakoid transfer-domains that are required for translocation of the proteins across the thylakoids.

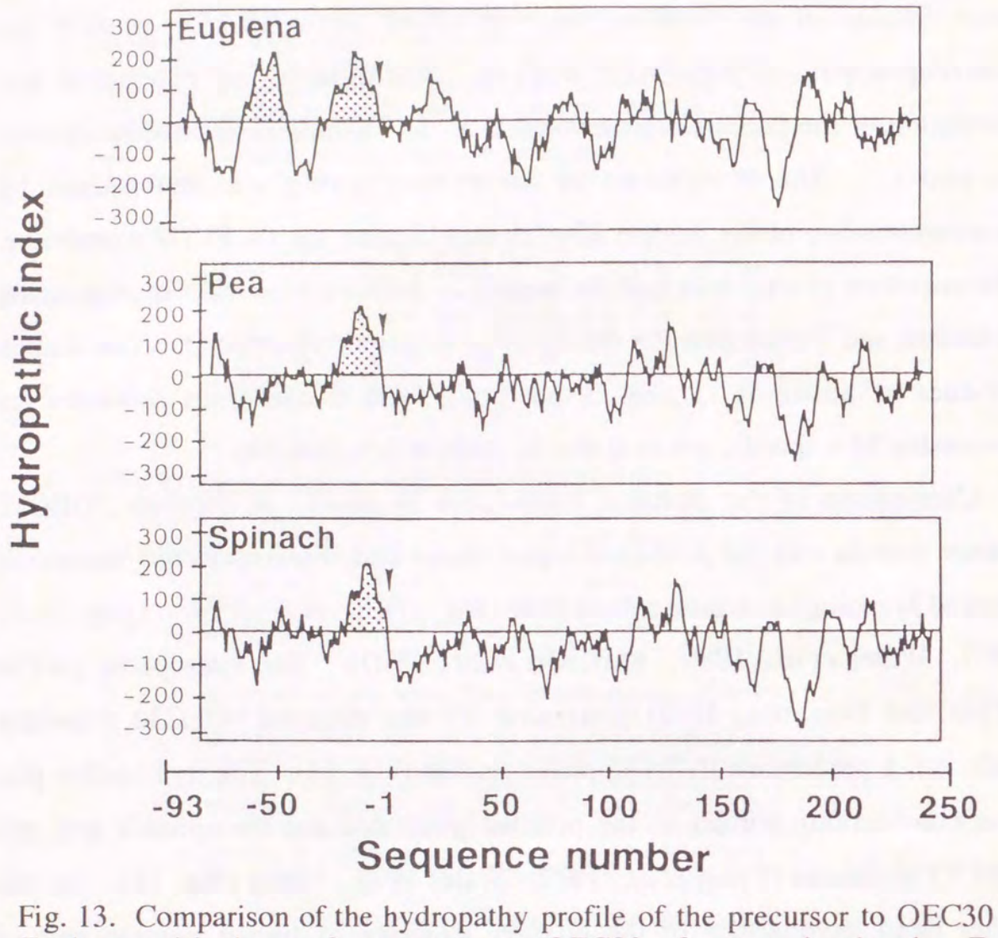

Fig. 13. Comparison of the hydropathy profile of the precursor to OEC30 of *Euglena* with those of precursors to OEC33 of pea and spinach. Each hydropathy plot, with a window size of eleven consecutive residues, is plotted against the residue number. The shadowed areas indicate the hydrophobic domains in the presequences. The arrowheads show the cleavage sites of presequences.

Gene copy number.

For analysis of the number of copies of the gene, genomic DNA from *Euglena* was digested by *Hind* III and *Kpn* I, blotted onto a membrane and hybridized with the ^{32}P-labelled clone of OEC30 as probe. Although there are no restriction sites specific for these enzymes in the gene for OEC30, several restriction fragments were observed on the blot (Fig. 14). This result suggests that OEC30 is encoded by a small multi-copy gene family. It should be noted

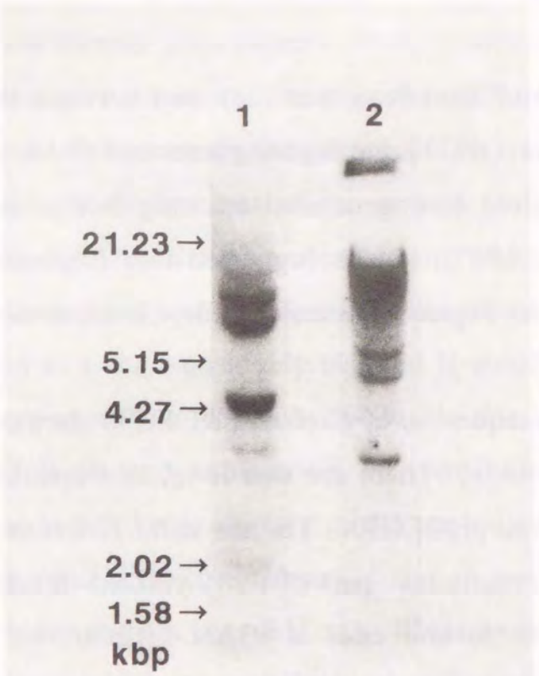

Fig. 14. Genomic Southern analysis of the gene for OEC30. Genomic DNA from *Euglena* was digested by *Hind* III (1) or *Kpn* I (2) and blotted onto a membrane. The membrane was allowed to hybridize to the same probe as that described in the legend to Fig. 11. The positions of molecular size markers are shown on the left.

that only one signal was seen in the Northern blot (Fig. 11), and several signals were seen in the Southern blot. This possibly means that all the additional genomic copies are not expressed under these conditions, or that several genes yield mRNA of the same size. It may be important to understand the regulation of the gene expression under various growth conditions.

Discussion

The high degree of homology was indicated between the mature protein sequences of *Euglena* OEC30 and higher plants and *Chlamydomonas* OEC33. From the genealogical tree generated by neighbor-joining method (Fig. 15)(Saitou and Nei, 1987), it was suggested that *Euglena* OEC30 mature protein had the higher degree of homology for *Chlamydomonas* OEC33 than higher plants OEC33.

However, the presequences of *Euglena* preOEC30 showed the considerably low degree of homology. There are two long, hydrophobic domains in the presequence of *Euglena* preOEC30. The one at the C-terminus, from 69Ala to 93Ala, apparently constitutes part of the thylakoid-transfer domain, which probably starts at 48Ala and ends at 93Ala. It is known that a processing

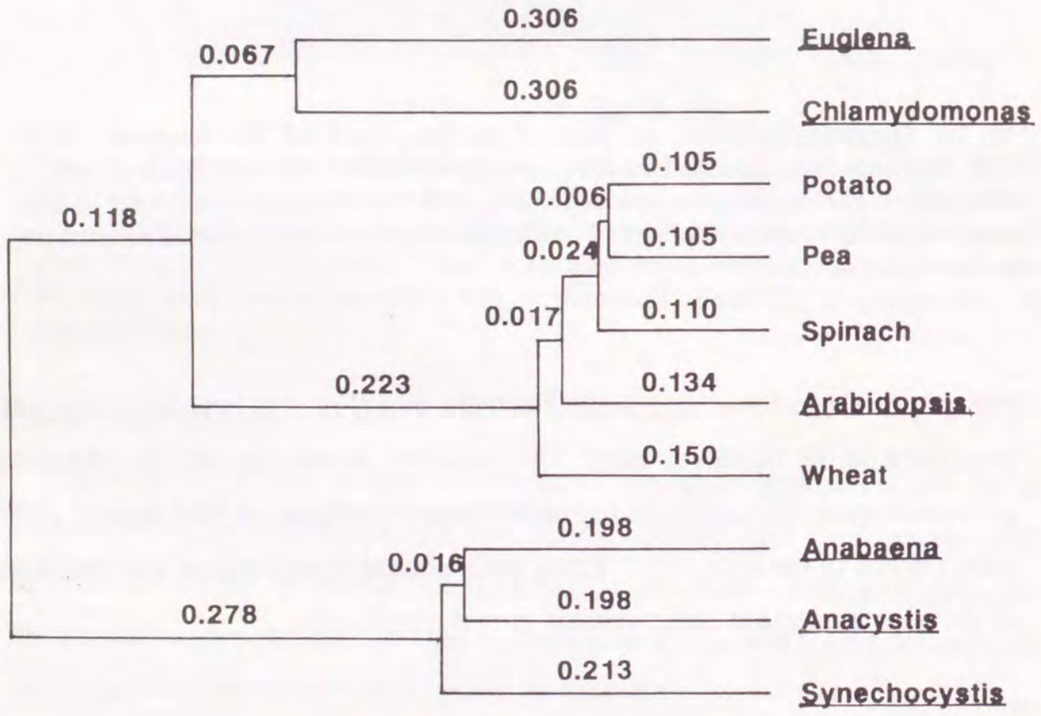

Fig. 15. The genealogical tree generated by neighbor-joining method.

protease in the thylakoids recognizes the consensus sequence Ala-X-Ala (von Heijne *et al.*, 1989) and cleaves presequences from mature proteins at the site indicated by the downward arrow. The sequence of 91Ala-92Asn-93Ala in OEC30 corresponds to this consensus sequence. From an analysis of the amino acid sequence, the N-terminus of the mature protein was shown to correspond to 94Ala. The thylakoid-transfer domain, which is homologous to the signal peptide (Smeekens and Weisbeek, 1988, von Heijne, 1985, von Heijne *et al.*, 1989) has a short positively charged N-terminal domain, which corresponds to the sequence from 48Ala to 68Glu. In this N-terminal domain, there are four positively charged amino acids. It contains a positively charged N-terminal region, a hydrophobic domain in the central region and a signal peptidase-processing motif at the C terminus.

Another hydrophobic domain from 33Trp to 47Ala may comprise part of a envelope transfer signal which we tentatively identified as the sequence from 1Met to 47Ala. The sequence 45Val-46Gly-47Ala-48Ala is homologous to the sequence proposed for the consensus cleavage site, (Ile/Val)-X-(Ala/Cys) (Ala), where the downward arrow indicates the cleavage site (Gavel and Heijne, 1990). No homology has been identified in the amino acid sequences of the envelope transfer signals examined to date. In general, envelope transfer signals have not been found to be particularly hydrophobic, being rich in serine and threonine and lacking tyrosine and negatively charged amino acids (de Boer and Weisbeek, 1991). Such features are not apparent in the corresponding sequence from *Euglena*. Instead, the sequence from 1Met to 47Ala was similar to a signal sequence, containing a positively charged N-terminal region, a hydrophobic core, and a more polar C-terminal region (von Heijne, 1985, 1988)(Fig. 16). However, like the N-terminal extension of *Euglena* preLHCPII, preSSU and prePBGD, the N-terminal signal peptide-like domain of preOEC30, in particular, a positively charged N-terminal region from 1Met to 32Asn is significantly longer than but structurally similar to most eukaryotic

```
 . +                  +     –  –     –   .  .              .
MSRVNLAAPA NHVVPRAAQE EVQGEYQTAS NNWAVAAMAS AGAAVGAAVL

  +++  .  .   –   +– –  –            +                .
AMRRRATNTY EAIREDPEAV LAGAGRAMGA ALIGAAVAGS ANA
```

Fig. 16. Structure of the transit peptide of *Euglena* preOEC30. Positively charged amino acids, negatively charged amino acids and hydroxy amino acids are shown as +, - and •, respectively. The long hydrophobic region is underlined.

signal peptides (von Heijne, 1986, 1988), including the 20-amino acid signal peptide found on the precursor to a *Euglena* ER-targeted protein (Gumpel and Smith, 1992).

The presence of a signal-peptide-like domain at the N-terminus of the presequence in preOEC30 suggests the involvement of ER- or ER-like membranes in the synthesis and/or transport of OEC30. However, this precursor protein was shown to be synthesized on free polysomes and post-translationally transported. The chloroplast envelopes of *Euglena* consists of three membranes, and the outermost membrane of the envelope has been suggested to be ER-like (Gibbs, 1981). A signal-peptide-like domain at the N-terminus of presequence in preOEC30 seems to be related to the structure of the chloroplast envelope of *Euglena*. The contact sites seem to be formed by association of the two inner membranes rather than all the three membranes. However, the author can not conclude that the outermost membrane is not involved at all in the formation of the contact site, because the outermost membrane was associated to the intermediate one in places. From these results described above, it is supposed that the outermost membrane of the chloroplast envelope is a significant barrier for the translocation of the precursors, or that the small amount of the contact sites formed by all the three membranes is a rate-limiting step for the process of import of precursor proteis in chloroplasts.

Here, two possibilities are suggested. The one is that *Euglena* preOEC30 probably has a tripartite presequence that consists of two signal-peptide-like domains at the N- and C-termini and another domain, which corresponds to a envelope transfer domain, between them. Transport across the *Euglena* chloroplast envelope may proceed in the two stages. The first stage is that N-treminal signal peptide-like domain is recognized by the system would be localized in the outermost membrane, and then the transport across the outermost membrane may occur. The second stage is the transport across the middle and inner membranes, which is supposed to be equal to chloroplast envelope transport in higher plants.

Another possibility is that preOEC30 probably has a bipartite presequence such as the precursor of thylakoid lumenal protein in higher plants. Because the N-terminal signal peptide-like domain of preOEC30 is significantly longer than most eukaryotic signal peptides (von Heijne, 1986,1988), only this signal may be imcompetent to transport across the ER-like outermost membrane. The sequence from 1Met to 47Ala may be the envelope transfer domain in the *Euglena* preOEC30. Transport across the *Euglena* chloroplast envelope probably proceeds in one stage using the contact sites formed by the three envelope membranes.

Summary

A cDNA clone for OEC30 in *Euglena* was isolated and characterized. The open reading frame of the cDNA encoded a polypeptide of 338 amino acids, which consisted of a long presequence of 93 amino acids and a mature polypeptide of 245 amino acids. Comparison of the deduced amino acid sequences of *Euglena* OEC30 mature polypeptide with the published higher plants and *chlamydomonas* sequences showed homologies of greater than 60%.

The hydropathy profile generated for the *Euglena* OEC30 sequence indicated the hydrophilic nature of the protein. The hydropathy plot was considerably similar to the profiles generated for the spinach and pea OEC33 sequences. On the other hand, comparison of the primary structure of presequence of *Euglena* preOEC30 with the presequences of higher plants and *chlamydomonas* indicated the low degree of homologies. The hydropathy profile of the precursor of *Euglena* OEC30 revealed a considerably greater difference between the N-terminal extensions of the transit peptides of *Euglena* and higher plants proteins. Two hydrophobic domains were identified in the presequence, in contrast to the presence of a single hydrophobic domain in the presequence of the corresponding proteins from higher plants. At the N- and C-terminal regions, respectively, of the presequence, a signal-peptide-like sequence and a thylakoid-transfer domain were identified. The presence of a long and unique presequence in the preOEC30 is probably related to the complexity of the intracellular processes required for the transport of the precursor protein in *Euglena*.

CHAPTER III

TRANSPORT OF preOEC30 TO CHLOROPLASTS IN HETEROLOGUS SYSTEMS

As described in Chapter I and II, the transit peptide of preOEC30, which are synthesized as a monomeric form on the free polysomes, is longer than that of corresponding proteins in higher plants (preOEC33) by about 10 amino acid residues, and the transit peptide includes two hydrophobic domains, and the processing of preOEC30 occured slowly. The C-terminal hydrophobic domain corresponds to the thylakoid transfer domain, while the N-terminal hydrophobic domain has a signal peptide-like structure. The N-terminal signal peptide-like domain is suggested to play an important role in the recognition of the import apparatus in the envelopes and translocation across the outermost (three) envelope membrane in *Euglena* chloroplasts (Shashidhara *et al.*, 1992).

In this chapter, the author examined whether the information in the transit peptide of the nucleus-encoded proteins of *Euglena* could be recognized by the protein transport apparatus in chloroplasts of higher plants. The author investigated the import of preOEC30 from *Euglena* into chloroplasts of spinach. Against our expectation, preOEC30 of *Euglena* was imported into spinach chloroplasts and transported to the lumen of thylakoid under the conditions where the precursor was simply incubated with the isolated chloroplasts in the presence of ATP in the light. The transit peptide of *Euglena* preOEC30 was further characterized using the chimeric precursor constructed from preOEC30 from *Euglena* and preOEC33 from pea. From the analyses of the kinetics of transport of these chimeric precursors, it was suggested that there are different between *Euglena* and higher plants in the transport system

of the precursors across the envelopes, although some similarities are expected in the process of recognition of precursor proteins on the surface of the envelopes. These results were discussed based on the difference of the structure of chloroplasts between *Euglena* and higher plants, which is related to the evolution of chloroplasts and also to the development of protein transport system in higher plants and algae.

Materials and Methods

Materials.

Spinach was obtained from local market. Pea (*Pisum sativum* L. var. Alaska) was grown in vermiculite for 12 days at 25°C under white fluorescent light. Restriction endonucleases were obtained from Takara Biochemicals (Japan) and New England Biolabs (U.S.A) and Toyobo (Japan). The cDNA encoding the precursor of porphobilinogen deaminase (PBGD) of *Euglena* was kindly provided by Dr. A. G. Smith, University of Cambridge.

Chimeric precursors.

A chimeric precursor protein, PE-E, consists of the mature part of *Euglena* OEC30, and the transit peptide of *Euglena* preOEC30 in which the N-terminal 44 amino acids were replaced by the N-terminal 32 amino acids of pea preOEC33. Consequently, the N-terminal hydrophobic domain in the presequence of *Euglena* preOEC30 was replaced by the envelope transfer signal of pea preOEC33. The EP-P is a chimeric protein, in which the N-terminal 32 amino acids in the transit peptide of pea preOEC33 was replaced by the N-terminal 44 amino acids of *Euglena* preOEC30. The EE-P is a chimeric protein, in which the mature part of pea OEC33 was fused with the tansit peptide from *Euglena* preOEC30. It has the thylakoidal processing site of

Euglena preOEC30. The PP-E is a chimeric protein, in which *Euglena* OEC30 mature protein was fused with the transit peptide of pea preOEC33. The protein contains the thylakoidal processing site of pea OEC33. The PE-P chimeric precursor protein consists of the mature part of pea OEC33 and the transit peptide of *Euglena* preOEC30 in which the N-terminal 44 amino acids were replaced by the N-terminal 32 amino acids of pea preOEC33. The PE-P has the thylakoidal processing site of *Euglena* OEC30.

Construction of plasmid.

The full-length cDNA coding for preOEC30 of *Euglena* was subcloned into the plasmid vector pBluescript II SK- (Strata Gene, U.S.A.) to yield the plasmid *Euglena*-preOEC30. The full-length cDNA coding for preOEC33 of pea (Wales, 1989) was subcloned into the pBluescript II KS+ (Strata Gene, U.S.A.) to yield the plasmid pea-preOEC33. Each chimeric clone to produce PE-E and EP-P was constructed as described below. The plasmid *Euglena*-preOEC30 was digested with a restriction endonuclease *Pvu* II. The ~1.2 kilobase fragment was isolated by gel electrophoresis (1.2% agarose). The plasmid pea-preOEC33 (Wales, 1989) was digested with *Ssp* I and *Aat* I. The ~2.3 kilobase fragment was isolated and ligated with the ~1.2 kilobase fragment from the plasmid *Euglena* -preOEC30 by the T4 DNA ligase (Takara, Japan) giving rise to the plasmid PE-E. To construct the plasmid EP-P, the full-length cDNA coding for preOEC30 of *Euglena* was subcloned into the pBluescript II KS+, and the full-length cDNA coding for preOEC33 of pea was subcloned inversely into the pBluescript II KS+. The plasmid *Euglena*-preOEC30 was digested with *Pvu* II. The ~260 base fragment was isolated. The plasmid pea-preOEC33 was digested with *Ssp* I and *Aat* I. The ~3.1 kilobase fragment was isolated and ligated with ~260 base fragment from the plasmid *Euglena*-preOEC30.

In order to generate the chimeric precursor proteins PP-E, EE-P and PE-P, the respective coding regions in the transit peptides and the mature proteins

were amplified separately by polymerase chain reaction (PCR) using the cDNA encoding *Euglena* preOEC30, pea preOCE33 and chimeric construct PE-E as the templates. A forward primer, 5'-CCGAGCTCGTCATGAGCCGCGTTAA-CTT-3', and a reverse primer, 5'-GCGAATTCGTCGTAAGTGAGGGACG-3', which were used for amplification of the sequence of the transit peptide in *Euglena* preOEC30 contained a *Sac* I restriction site and an in-frame *Eco* RI site, respectively, so that the 101-leucine residue (8 amino acid residues downstream of the mature processing site) of *Euglena* preOEC30 was substituted by a phenylalanine residue. The primers for amplification of the mature part of *Euglena* OEC30, which correspond to the animo acid sequence from 101-phenylalanine to the C-terminus lysine, were 5'-CGGAATTCCAGT-CCCTGTCCTACTTGGA-3' and 5'-GCCTCGAGTGCACGGTTGAAAGAGT-TTA-3', which contained an in-frame *Eco* RI site and a *Xho* I site, respectively. The primers for amplification of the transit peptide sequence of pea OEC33 were 5'-AAGAGCTCATGGCAGCCTCACTTCAA-3' and 5'-GCGAATTCGT-CGAAAGTTAGCCTCT-3'. They contained a *Sac* I site and an in-frame *Eco*RI site, respectively, so that the 93-isoleucine residue (12 amino acid residues downstream of the mature processing site) of pea preOEC33 was substituted by a phenylalanine residue. The primers for amplification of the mature part of pea OEC33, which correspond to the animo acid sequence from 93-phenylalanine to the C-terminus serine, were 5'-CGGAATTCCAAAGCAAAA-CATACTTG-3' and 5'-GTCTCGAGAAGCTATCTATGATTCAA-3', which contained an in-frame *Eco* RI site and a *Xho* I site, respectively. The primers for amplification of the transit peptide of the chimeric precursor protein PE-E were 5'-AAGAGCTCATGGCAGCCTCACTTCAA-3' and 5'-GCGAATTCGT-CGTAAGTGAGGGACG-3', which contained a *Sac* I site and an in-frame *Eco*RI site, respectively. PCR amplification was performed by using a *Taq* polymerase (Promega, U.S.A.) with 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min (for the last cycle, 5 min) at 72°C. The PCR products were isolated

by gel electrophoresis (1.5% agarose). Each PCR product of mature part of the protein was digested with *Xho* I and *Eco* RI, and ligated with an appropriately digested pBluescript II KS+. The ligated pBluescript II KS+ was then digested with *Sac* I and *Eco* RI, and ligated with PCR products of the transit peptide sequences, which were digested by *Sac* I and *Eco* RI, respectively. DNA sequences were analyzed by dideoxy chain-termination method using BcaBEST™ Dideoxy Sequencing Kit (Takara, Japan).

In vitro transcription and translation.

Plasmids carrying DNA fragments encoding either authentic or chimeric precursor proteins were linearized downstream of the inserted DNA fragment. Transcription reactions were performed using either T3 (Ambion, U.S.A.) or T7 RNA polymerase (Takara, Japan), depending on the orientation of the insert within the vector. The transcripts were translated *in vitro* in a rabbit reticulocyte lysate system (Promega, U.S.A.). The translation mixture (50 ml) contained 35 ml of the rabbit reticulocyte lysate, 2 ml of the transcript aliquot, 4 ml of [^{35}S]methionine (37 MBq nmol^{-1}) (ICN, U.S.A.), and 1 ml of a mixture of the other amino acids. The translation mixture was incubated at 30°C for 90 min.

Chloroplast isolation from spinach and pea.

Intact chloroplasts were isolated from spinach and pea by a modification of the procedure described by Bartlett *et al.* (Bartlett *et al.*, 1982). A crude chloroplast preparation was obtained by centrifugation at 3,000 x *g* for 1 min. Intact chloroplasts were then purified on a step-wise Percoll gardient (20, 50 and 80% v/v) by centrifugation at 8,000 x *g* for 10 min. Intact chloroplasts removed from the gradient were diluted 3 fold with import buffer (50 mM Hepes-KOH, 0.33 M sorbitol and 5 mM $MgCl_2$, pH 8.0), and centrifuged at 1,500 x *g* for 2 min. The chloroplast pellet was washed once with import

buffer and resuspended in import buffer to a concentration eqivalent to 2 mg chlorophyll per ml. Chl was measured according to the method of Arnon (Arnon, 1949).

Import assay.

Import assays with spinach and *Euglena* chloroplasts were performed according to Cline *et al.* (Cline, 1985). Import reactions were carried out under the illumination with white light for 20 - 30 min at 25°C in a reaction mixture (300 ml) containing 10 - 30 ml translation mixture, which contains radiolabeled protein (150,000 cpm), 10 mM ATP, 10 mM $MgCl_2$, and chloroplasts equivalent to 100 μg of chlorophyll. After the reaction, intact chloroplasts were reisolated on 40% Percoll cushions. The chloroplasts were treated with thermolysin (100 μg/ml) on ice for 15 min where indicated (Cline *et al.*, 1984). Chloroplasts recovered were lysed on ice by incubation in a solution of 10 mM Hepes-KOH, 10 mM $MgCl_2$, pH 8.0 at 1.0 mg chlorophyll/ml for 5 min. Membranes and soluble fractions were separated by centrifugation at 3,500 x *g* for 5 min. Thylakoid fraction was treated with thermolysin (100 μg/ml) for 15 min on ice. Protein fractions were resuspended in a loading buffer (2.5% SDS, 10% sucrose, 5% β-mercaptoethanol, 2 mM EDTA, 0.1 M Tris-HCl, 0.004% Coomassie Brilliant Blue, pH 6.8) and subjected to SDS-PAGE using 12.5% acrylamido gels. The protein bands were analyzed by a Fuji Imaging Analyzer BAS 2000. The radioactivity of the protein bands were also quantitated by BAS 2000.

Hydropathy analysis.

Hydropathy analysis of the amino acid sequence was carried out by the method of Kyte and Doolittle (Kyte and Doolittle, 1982) with a window size of 11 amino acids.

Results

Transport of pea preOEC33 and *Euglena* preOEC30 into spinach chloroplasts.

The pea preOEC33 was efficiently imported into pea and spinach chloroplasts in the presence of ATP and under the illumination with white light (Fig. 17). Mature pea OEC33 was located correctly in the lumen of the

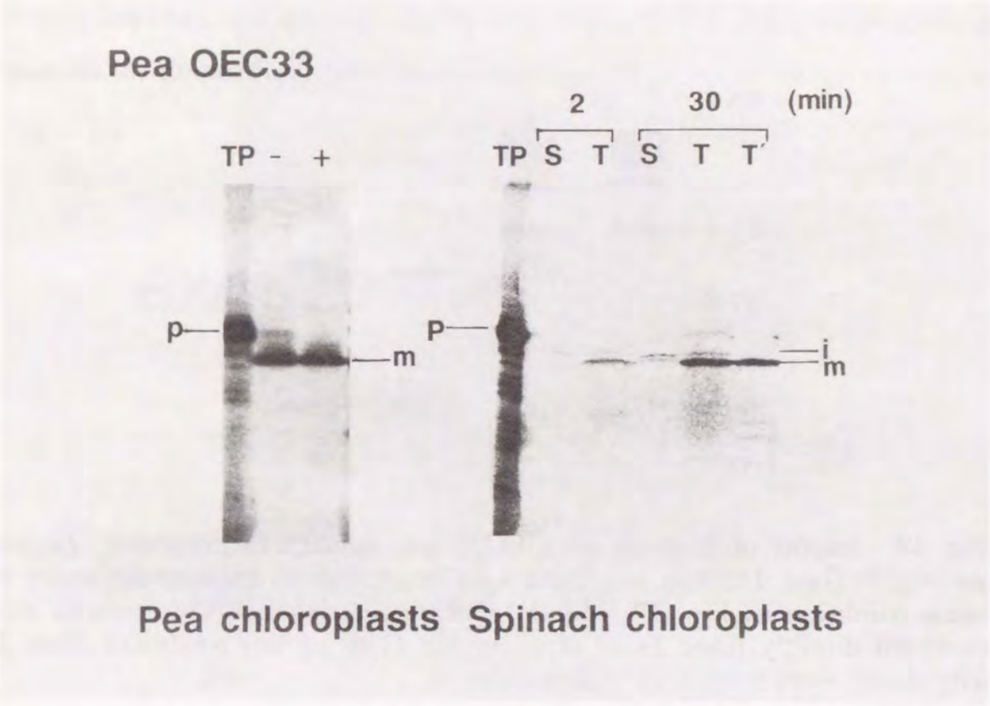

Fig. 17. Import of pea preOEC33 into pea and spinach intact chloroplasts. Pea preOEC33 (lane TP) was incubated with isolated intact pea or spinach chloroplasts in the presence of 10 mM ATP in the light at 25°C. After 30 min of incubation, reisolated pea chloroplasts were analysed directly (lane -) or after thermolysin (100 μg/ml) treatment (lane +)by SDS-polyacrylamide gel electrophoresis and by a Fuji Imaging Analyzer BAS 2000. After 2 min and 30 min of incubation, reisolated spinach chloroplasts were fractionated into stroma (lane S) and thylakoids. One fraction of the thylakoids was treated prior to analysis with thermolysin (lane T'), and the other was non treated (lane T). The positions of the precursors (p), intermediates (i), and mature protein forms (m) are indicated.

thylakoids as judged by the resistance to the externally added protease. As the efficiency of transport of the precursor to spinach chloroplasts and that to pea chloroplasts were almost the same (Fig. 17), and as spinach was readily available, the author used spinach chloroplasts in the following experiments of protein transport *in vitro*.

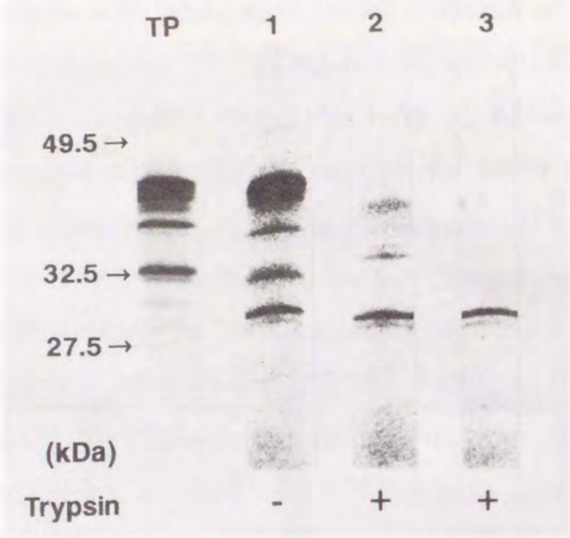

Fig. 18. Import of *Euglena* preOEC30 into spinach chloroplasts. *Euglena* preOEC30 (lane TP) was incubated with intact spinach chloroplasts under the same condition as Fig. 17. After incubation, reisolated chloroplasts were analysed directly (lane 1) or after trypsin (100 μg/ml) treatment (lane 2). Thylakoids were treated by trypsin (lane 3).

Previously, Shashidhara *et al.* showed that the precursor to *Euglena* PBGD was efficiently imported into *Euglena* chloroplasts. At the same time, they showed that the precursor is able to bind to pea chloroplasts without being imported into the chloroplasts at all (Shashidhara *et al.*, 1992). These results indicate that the chloroplasts from higher plants and *Euglena* do not have the same import apparatus, although some similarities are expected in the process of recognition of precursor proteins on the surface of the envelopes. In

heterologous systems of protein transport, where combination of precursors and chloroplasts originated from different plant species is used, the efficiency of transport is limited by the structure of the precursor molecules, and by the structure and function of the transport machineries in chloroplasts including the receptor proteins and the channel-forming proteins in the membranes, cytosolic factors and stromal factors. The author expected that *Euglena* preOEC30 is not imported into spinach chloroplasts by analogy with the results of Shashidhara *et al.* However, against our expectation, *Euglena* preOEC30 was not only imported into spinach chloroplasts, but also was transported across the thylakoid membranes and fully maturated (Fig. 18).

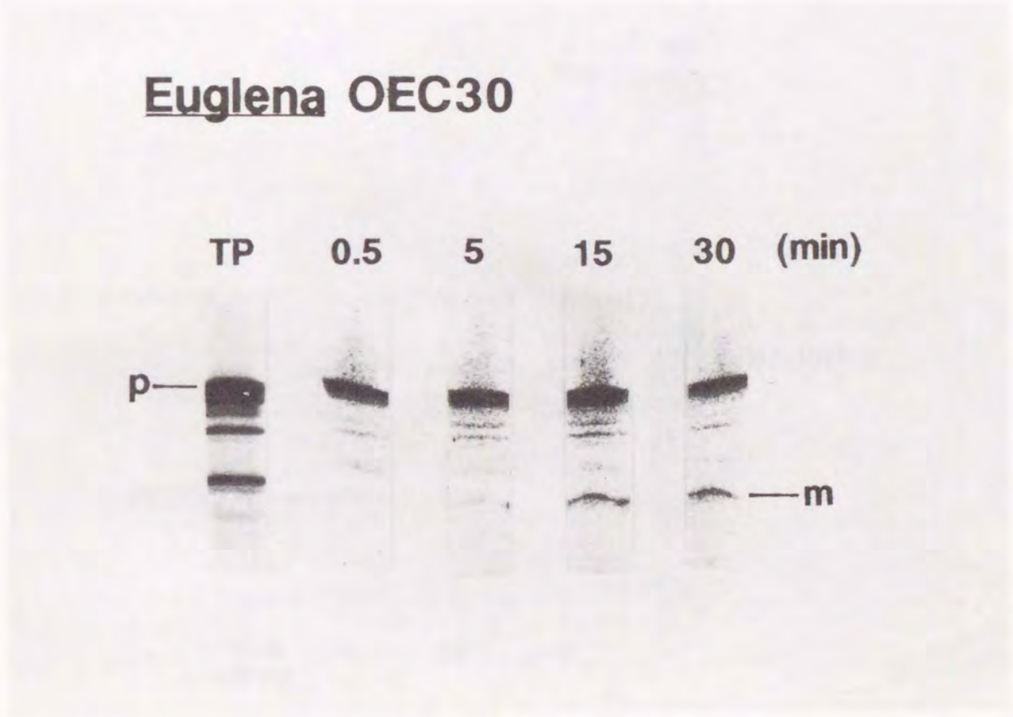

Fig. 19. Import time course of *Euglena* preOEC30. *Euglena* preOEC30 was incubated with spinach intact chloroplasts for periods given above the lanes (in min). After incubation, samples were analyzed directly. Symbols are the same as described in the legend to Fig. 17.

A significant difference between the transport of pea preOEC33 and *Euglena* preOEC30 into spinach chloroplasts is that the latter process proceeded much slowly. Under the conditions where saturating amounts of precursors were added in the reaction mixture, pea preOEC33 was transported into thylakoid lumen after 1 min of illumination, while mature OEC30 of *Euglena* was not detected in the chlorolpasts until 5 min of illumination (Fig. 19). As an excessive amount of radiolabeled precursor was required to demonstrate the transport of *Euglena* preOEC30 into spinach chloroplasts, apparently, the efficiency of import of *Euglena* preOEC30 into spinach chloroplasts is lower than that of pea preOEC33. As a large amount of rabbit reticulocyte lysate included in the translation mixture led to destruction of intact chloroplasts,

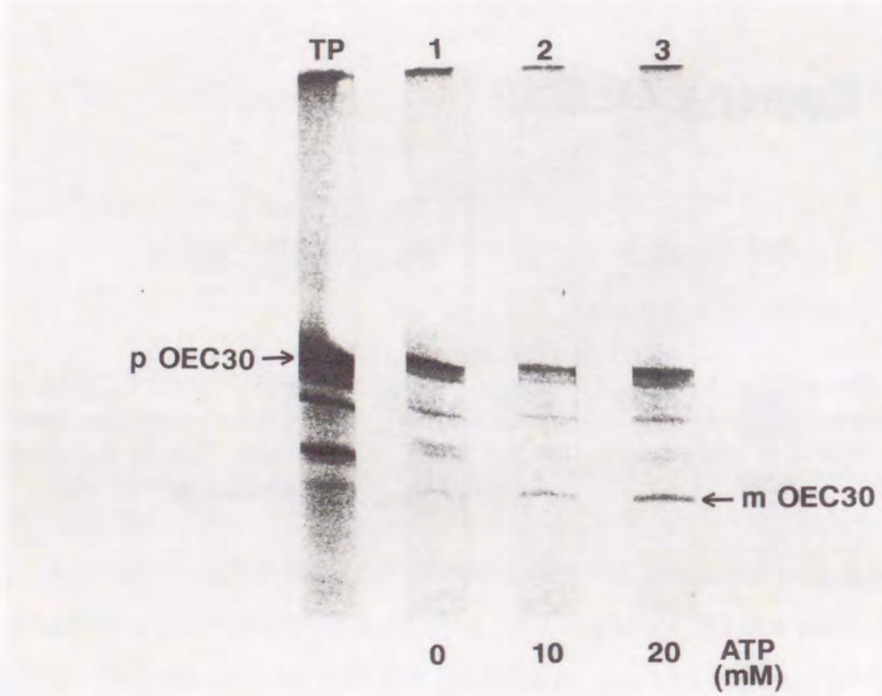

Fig. 20. Effect of added ATP on the import of *Euglena* preOEC30 into spinach chloroplasts. *Euglena* preOEC30 (lane TP) was incubated with intact chloroplasts and ATP at concentrations (in mM) given above the lanes in the light at 25°C for 30 min.

further analysis of the kinetics of protein transport was not carried out.

It was reported with *in vitro* import of proteins into chloroplasts from higher plants that a low concentration of ATP (5-100 μM) is necessary for the binding of the precursors to the envelope of chloroplasts, while a higher concentration of ATP (0.2-2 mM) is required for the import of the precursors into chloroplasts (Olsen, *et al.* 1989, Olsen and Keegstra, 1992). The author studied the requirement of ATP in the transport of *Euglena* preOEC30 into spinach chloroplasts (Fig. 20). Without added ATP, mature OEC30 was scarcely detected, and by the addition of 10 -20 mM ATP, the transport was almost saturated. Apparently, about 10 times larger amount of ATP is required

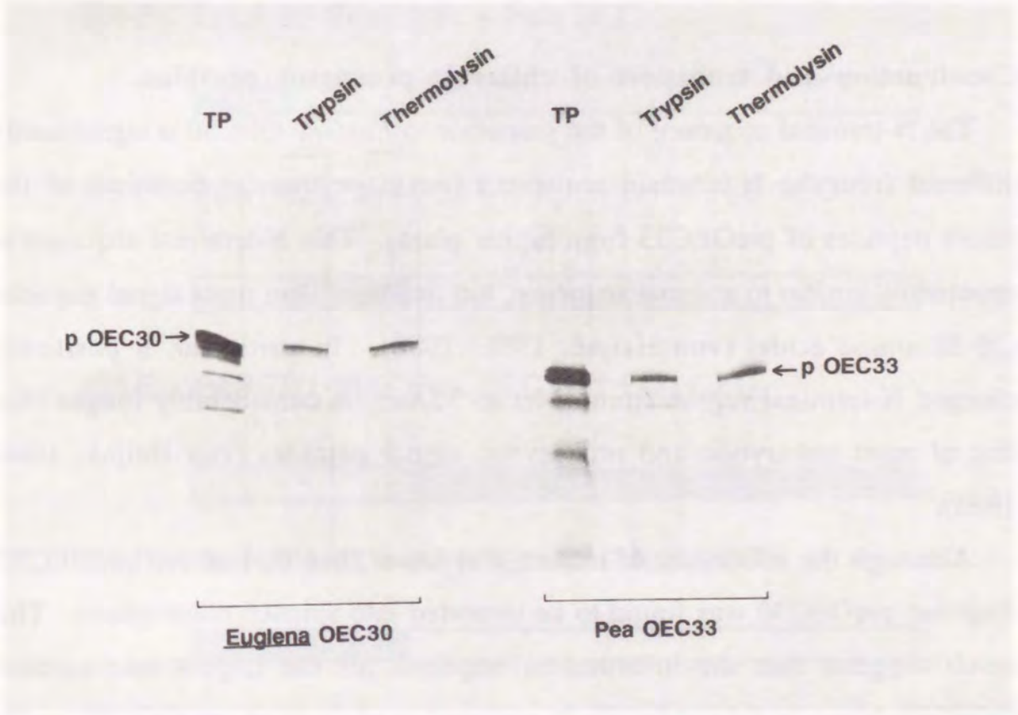

Fig. 21. Effects of protease pretreatment of chloroplasts on the import of pea preOEC33 and *Euglena* preOEC30. Intact spinach chloroplasts were treated with 0.5 mg/ml trypsin or thermolysin in the dark on the ice. After termination of proteolysis, chloroplasts were repurified on 40% Percoll cushions, washed with import buffer, and were then tested for their ability to import pea preOEC33 and *Euglena* preOEC30.

for the transport of *Euglena* preOEC30 to spinach chloroplasts, compared with the protein transport with higher plants systems.

To examine if the import of *Euglena* preOEC30 into spinach chloroplasts is mediated by a proteinaceous receptor, the chloroplasts pretreated with trypsin or thermolysin were used for the import experiments (Cline *et al.*, 1985)(Fig. 21). With the protease-treated chloroplasts, the binding of the precursor proteins to chloroplasts was significantly reduced and little protein was detected as the mature proteins. These results indicate that the transit peptide of *Euglena* preOEC30 was recognized by specific proteinaceous receptors on the envelope of spinach chloroplasts, and that transport of preOEC30 through the envelopes was mediated by the receptors.

Construction and transport of chimeric precursor proteins.

The N-terminal sequence of the precursor to *Euglena* OEC30 is significantly different from the N-terminal sequences (envelope transfer domains) of the transit peptides of preOEC33 from higher plants. This N-ternimal sequence is structurally similar to a signal sequence, but is larger than most signal peptides (20-30 amino acids) (von Heijne, 1986, 1988). In particular, a positively charged N-terminal region from 1Met to 32Asn is considerably longer than that of most eukaryotic and prokaryotic signal peptides (von Heijne, 1986, 1988).

Although the efficiency of import was lower than that of pea preOEC33, *Euglena* preOEC30 was found to be imported into spinach chloroplasts. This result suggests that the information required for the import into spinach chloroplasts may reside in the the transit peptide of *Euglena* preOEC30, or the whole sequence of the precursor.

Therefore, to characterize the transit peptide in *Euglena* preOEC30, the author has constructed chimeric precursor proteins from *Euglena* preOEC30

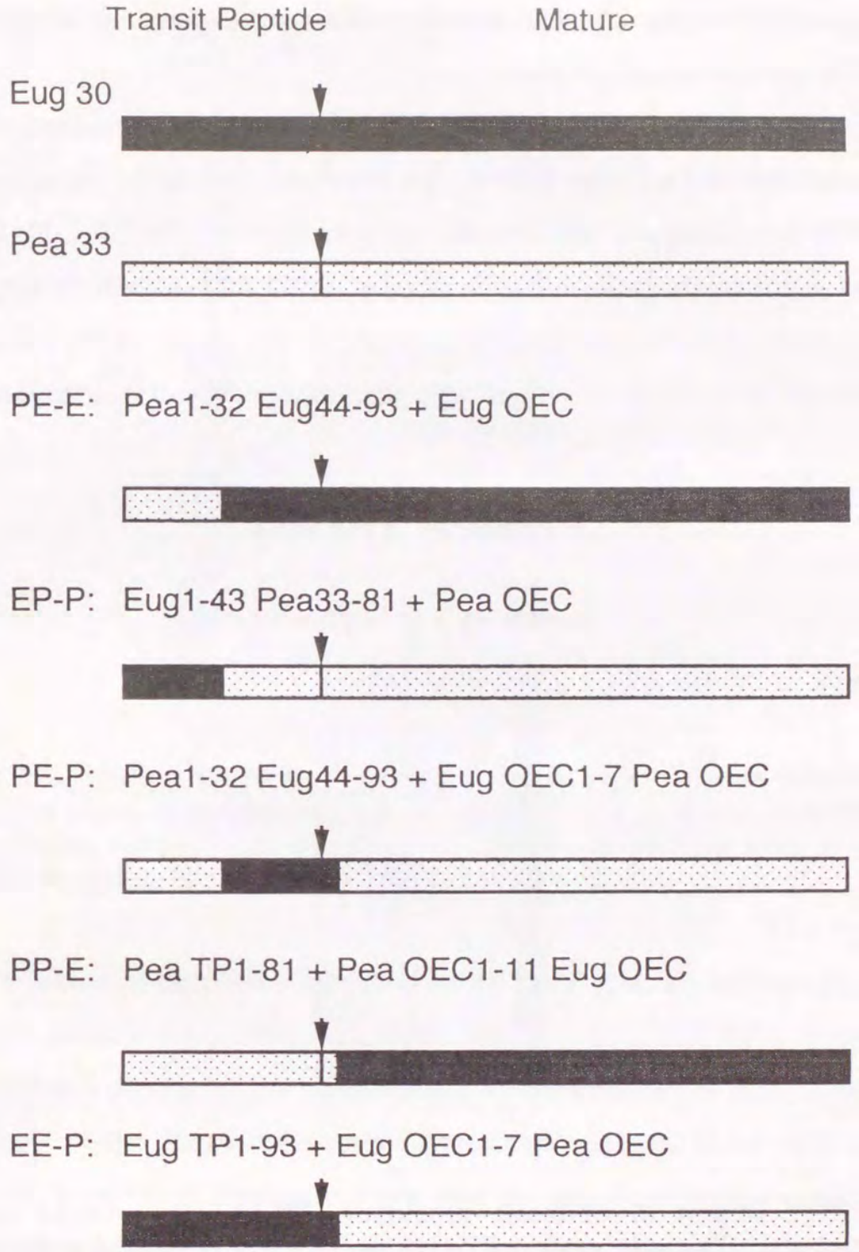

Fig 22. Construction schemes for these chimeric precursors. The details of the construction of these chimeric precursors are described in Materials and methods. The labels within the bars depict the origin of that segment. The arrowhead represents the cleavage/processing site. TP, transit peptide ; OEC, mature part of the 33 kDa oxygen-evolving protein.

and pea preOEC33 (Fig. 22), and carried out transport experiments using these precursors and spinach chloroplasts.

Firstly, the author investigated the import of a chimeric precursor PP-E, which consisted of the entire transit peptide of pea preOEC33 including the thylakoidal processing site and the mature part of *Euglena* OEC30. PP-E was imported as efficiently as the authentic pea preOEC33 into spinach chloroplasts, and the mature OEC30 was located correctly in the lumen of thylakoids as judged by resistance to the treatment with thermolysin (Fig. 23). Illumination

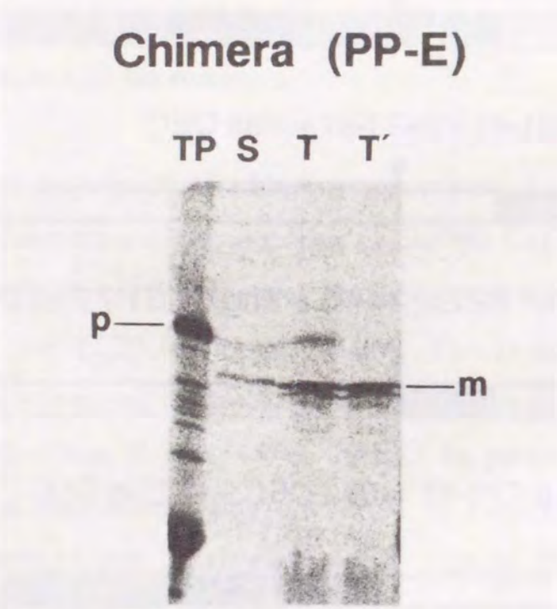

Fig. 23. Import of chimeric precursor (PP-E) into isolated spinach chloroplasts. Chimeric protein PP-E (lane TP) was incubated with isolated intact spinach chloroplasts under the same condition as Fig. 17. Symbols are the same as described in the legend to Fig. 17.

for 30 sec-1 min at 25°C was enough for the detection of the mature protein of *Euglena* OEC30 (Fig. 24). Fig. 24 shows that in a time course experiment a processing intermediate could be observed. The kinetics of appearance and

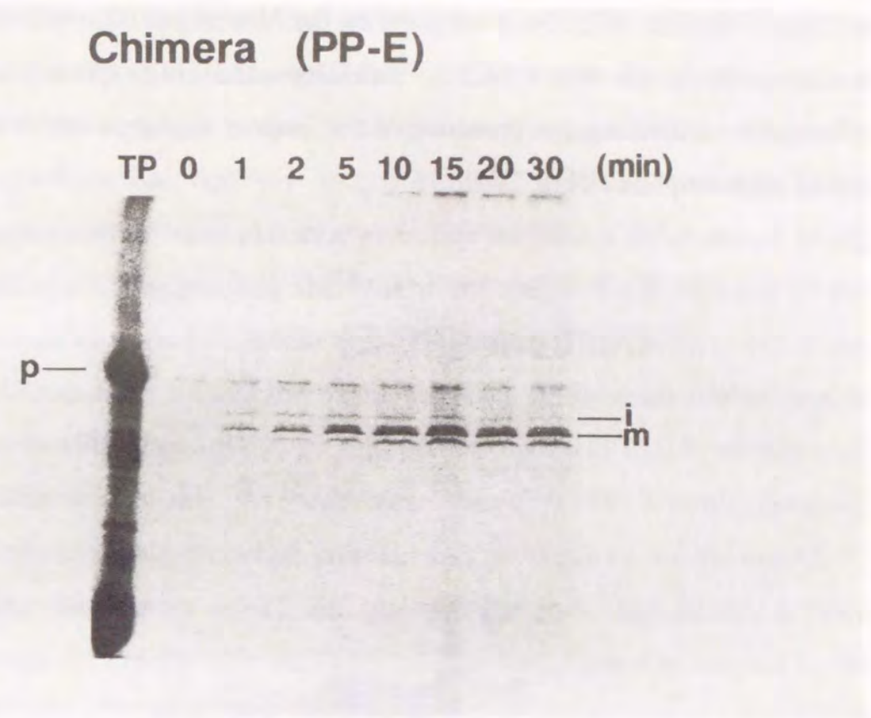

Fig. 24. Import time course of PP-E. PP-E (lane TP) was incubated with spinach intact chloroplasts for periods given above the lanes (in min). After incubation, samples were analyzed directly. Symbols are the same as described in the legend to Fig. 17.

disappearance of the processing intermediate of PP-E was similar to that of pea OEC33 (Bauerle *et al.*, 1991). It may be not surprising that the presence of the whole transit peptide of pea precursor enable the transport of mature protein of *Euglena* to the lumen of spinach thylakoids, because many authors have reported transport of foreign proteins with the aid of authentic transit peptides (Schreier *et al.*, 1985, Lubben and Keegstra, 1986, Ko and Cashmore, 1989, Meadows *et al.*, 1989). The signal that directs the foreign proteins to the chloroplasts and that imports into the chloroplasts is located at the N-terminal part of the transit peptide of pea preOEC33 (Ko and Cashmore, 1989). A chimeric protein PE-E, in which the N-terminal 44 amino acids of the transit

peptide of *Euglena* OEC30 is replaced by the N-terminal 32 amino acids of the transit peptide of pea OEC33, was imported into spinach chloroplasts efficiently, confirming the presence of the import signal in the N-terminal 32 amino acids-sequence (Fig. 25).

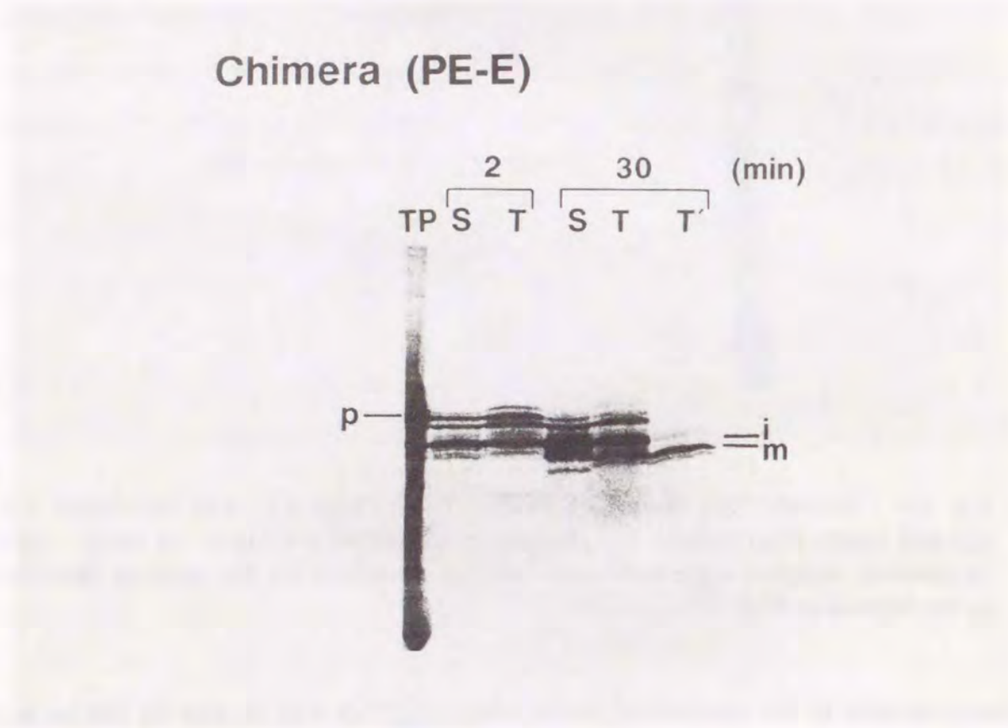

Fig. 25. Import of chimeric precursor (PE-E) into isolated spinach chloroplasts. Chimeric protein PE-E (lane TP) was incubated with isolated intact spinach chloroplasts under the same condition as Fig. 17. Symbols are the same as described in the legend to Fig. 17.

A chimeric precursor, EE-P, which has the transit peptide of *Euglena* OEC30 and the mature part of pea OEC33, was bound to spinach chloroplasts but not imported into the chloroplasts (data not shown). The precursor bound to the chloroplasts were protease-sensitive, implying that they remained on the outside of the chloroplasts. Another chimera, EP-P, which contains the N-terminal sequence (44-amino acids) from the transit peptide of *Euglena*

OEC30, the C-terminal part of the transit peptide of pea OEC33, and the mature pea OEC33, was neither bound to or transported across the envelope membranes. Although the author is unable to eliminate the possibility of unsuitable combinations between transit peptide parts and mature parts, structural aberrations of these chimeric precursor molecules (Clausmeyer *et al.*, 1993), these results suggest that the N-terminal only 44 amino acids of the transit peptide of *Euglena* preOEC30 do not possess the capability to direct pea OEC33 to chloroplasts, and that the whole sequence of *Euglena* transit peptide is competent to direct pea OEC33 to chloroplasts but insufficient for transport of the protein across the envelope membranes. The conformational information of the whole precursor proteins may be required for the import.

Following the import of PE-E, most of the imported products were processed to the intermediate form. This intermediate form was located in the stromal compartment and the membrane fraction, and represented ~ 60 - 70% of the imported products (Fig. 25, lane S and T). ~ 30 - 40% of the imported products were processed to the mature form and localized in the thylakoid lumen (Fig. 25, lane T'). The intermediates bound quite firmly to the thylakoid membrane were protease-sensitive. The intermediates were able to attach to the outside of the thylakoid membrane, but were unable to transport across the thylakoid membrane and to translocate in the lumen. A chimeric precursor protein PE-P, which has the same transit peptide as a PE-E precursor protein, was also imported into spinach chloroplasts efficiently, but most of the imported products were accumulated as the intermediate form in stroma (data not shown). These results indicated that as the efficiencies of transport of PE-E and PE-P across the thylakoid membrane were lower than those of transport of these chimeric precursors across the envelope membranes, the intermediates were accumulated in the stromal compartment. It was suggested that the putative thylakoid transfer domain of *Euglena* preOEC30 was capable of

mediating translocation the protein across the thylakoid membrane of spinach chloroplasts, but that the efficiency was lower than that of higher plants.

Discussion

The precursor of *Euglena* preOEC30 was found to be imported into spinach chloroplasts and to be translocated to the lumen as the mature form. This result is the first demonstration of transport of the precursor of nucleus-encoded protein destined for the chloroplast of *Euglena* into chlroplasts of higher plants. The characteristics of import of *Euglena* preOEC30 into spinach chloroplasts were that the efficiency was lower than that of pea preOEC33, that about 10 times larger amount of ATP was reqired for the transport, compared with higher plants systems, and that without added ATP, the transport was scarcely detected even in the light. Translocation of *Euglena* preOEC30 across the envelopes of spinach chloroplasts was shown to be mediated by a proteinaceous receptor. This result suggested that the transit peptide and/or the mature part of *Euglena* preOEC30 possessed the capability to target spinach chloroplasts and to recognize the receptor in the envelope membrane of spinach chloroplasts. However, it was unclear whether *Euglena* preOEC30 and pea preOEC33 were recognized by the same proteinaceous receptor in the envelope membranes of spinach chloroplasts.

Euglena chloroplasts have envelopes consisting of triple membranes (Gibbs, 1970, Lefort-Tran *et al.*, 1980), in contrast with the double membrane of higher plants and green algae. The transit peptides of all precursors of *Euglena* chloroplast proteins examined to date have N-terminal hydrophobic domains, which are similar to the signal sequence. In general, this N-terminal hydrophobic domain is suggested to be required for translocation of the protein across the outermost envelope membrane of *Euglena* chloroplasts. Namely,

transport across the *Euglena* chloroplast envelope may proceed in the two stages. The first stage is that N-treminal signal peptide-like domain is recognized by the system would be localized in the outermost membrane, and then transport across the outermost membrane may occur. The second stage is transport across the middle and inner membranes, which is supposed to be equal to chloroplast envelope transport in higher plants. However, as the N-terminal signal peptide-like domain of *Euglena* preOEC30 is significantly longer than most eukaryotic signal peptides (von Heijne, 1986, 1988), only this signal may be imcompetent to transport across the ER-like outermost membrane. It is supposed that the N-terminal signal peptide-like domain may be required for the only recognition of transport receptor system would be localized in the outermost membrane, and then transport of preOEC30 across the *Euglena* chloroplast envelope probably proceeds in one stage using the contact sites formed by the three envelope membranes. It was expected that some similarities may reside in the translocation system of proteins across the *Euglena* and higher plants chloroplast envelopes.

The author characterized using some chimeric proteins the transit peptide of *Euglena* preOEC30. The N-terminal hydrophobic sequence (the first 44-amino acids) itself did not possess the capability to direct the protein to spinach chloroplasts. The whole transit peptide of *Euglena* preOEC30 was competent to direct the protein to chloroplasts and to bind to the envelope membranes but was insufficient for transport the protein across the envelope membranes. The suitable combination of transit peptides and mature proteins may be required for the import into spinach chloroplasts (Table II).

From the import experiments with PE-E and PE-P, the putative thylakoid transfer domain of *Euglena* preOEC30 was shown to be capable of mediating translocation the protein across the thylakoid membrane of spinach chloroplasts, and to possess the structural and fuctional similarity to the thylakoid transfer domain of higher plants. Moreover, there is a possibility that the thylakoid

transfer apparatus in the thylakoid membrane may be similar between *Euglena* and higher plants chloroplasts. However, as the intermediates accumulated in stroma and bound to the thylakoid membranes were detected in the import experiment with PE-E and PE-P, some factors may be required for the efficient translocation to the thylakoid lumen.

Table II. Efficiencies of translocation of chimeric precursor polypeptides across envelope and thylakoid.

Precursor protein	Translocation across envelope	Translocation across thylakoid
TP M		
Eug 30	+	+
Pea 33	+++	+++
PE-E	+++	+
EP-P	-	(+++)
PE-P	+++	+
PP-E	+++	+++
EE-P	-	(+)

From these results described above, it is suggested that the similar mechanism exists in protein translocation across the thylakoid membrane between *Euglena* and higher plants which are evolutionary distant from each other. *Euglena* transit peptides appear to bind specifically to spinach

chloroplast envelopes. This suggests that the signal-receptor interaction system in the outer envelope membranes of *Euglena* and higher plants are radically similar. In view of the many peculiarities of *Euglena* chloroplasts and their proteins, it might be expected that there are several differences in translocation across the envelope membranes.

The OEC33 thylakoid transfer signal resemble structurally and functionally the signal sequence that is required for the translocation of proteins across the endoplasmic reticulum (ER) and across the plasma membrane of bacteria (von Heijne *et al.*, 1989). Halpin *et al.* have shown that the reaction specificities of the thylakoidal processing peptidase and *Escherichia coli* leader peptidase were essentially identical (Halpin *et al.*, 1989). Gene homologues of *sec*A and *sec*Y, which encode components of the bacterial protein export apparatus, are present in the plastid genomes of several species of alga (Douglas, 1992, Scaramuzzi *et al.*, 1992, Flachmann *et al.*, 1993, Valentin, 1993). Robinson *et al.* have shown that the SecA inhibitor, azide reversibly blocked the translocation of OEC33 and plastocyanin across the chloroplast thylakoid membrane (Knott and Robinson, 1994). They have suggested the possibility that the OEC33 and plastocyanin translocation system across the thylakoid membrane may operate by a *sec*-type mechanism. It is suggested that the mechanism for thylakoidal protein (OEC33 and plastocyanin) translocation may possess a common system from cyanobacteria to higher plants.

The mechanism for import into chloroplasts has been suggested to be added to the original thylakoid routing, which the cyanobacterial-type progenitor of higher plant chloroplasts would have possessed, after the initial endosymbiotic event (Hageman *et al.*, 1990). If *Euglena* chloroplasts evolved by a secondary endosymbiosis of a eukaryotic alga (Gibbs, 1981), to transport the nucleus-encoded proteins across the remained outermost membrane, the chloroplast import mechanism of a eukaryotic alga probably would have to be modified.

Summary

The precursors of nucleus-encoded proteins of *Euglena* have N-terminal hydrophobic domains in their transit peptides generally, which is suggested to be required for translocation of the proteins across the envelopes of chloroplasts consisting of triple membranes. To date, transport of these precursors to the chloroplasts of higher plants has not been reported. The author carried out transport of *Euglena* OEC30 into spinach chloroplasts to examine the signal-receptor interaction between these plant species which are evolutionary distant from each other. *Euglena* OEC30 was imported into spinach chloroplasts. In the study of protein transport with chimeric precursor proteins using *Euglena* preOEC30 and pea preOEC33, the author demonstrated that the N-terminal hydrophobic sequence (the first44-amino acids) of *Euglena* preOEC30 did not recognize the surface receptor of spinach envelopes, but within the whole transit peptide there was a signal which enables the precursor to bind to the envelopes. It was also suggested that the transit peptide of *Euglena* preOEC30 itself is not sufficient for the tranlocation of the protein across envelopes, and that an appropriate conformation of the precursor, which may be brought about by suitable combination of transit peptides and mature proteins, is necessary for transport of proteins into higher plants chloroplasts. The putative thylakoid transfer domain of transit peptide of *Euglena* preOEC30 was shown to be capable of mediating translocation the protein across the thylakoid membrane of spinach chloroplasts, but that the efficiency was lower than that of higher plants. It is suggested that the similar mechanism exists in protein translocation across the thylakoid membrane between *Euglena* and higher plants which are evolutionary distant from each other.

CONCLUSION

Euglena gracilis is a unicellular alga and the chloroplasts of *Euglena* have envelopes consisting of triple membranes. The outermost membrane has been suggested to be derived from ER or from the plasma membrane of endosynbiont in the secondary endosymbiotic events. It has been reported that *Euglena* has specific features in the structure of precursors of the nuclear-encoded chloroplast proteins.

In chapter I, the precursor of the nuclear-encoded 30 kDa protein associated with PS II (preOEC30) in *Euglena* chloroplast was identified as a 45 kDa protein by immunoprecipitation of *in vitro* translation products of mRNA and by a pulse-chase experiment. The preOEC30 was shown to have a longer transit peptide compared with the corresponding precursor of the OEC33 from higher plants. From the pulse-chase experiment, the presence of a large precursor and the slow processing observed here with the OEC30 of *Euglena* are probably related to the complexity in the processing of the protein in this organism.

In chapter II, *Euglena* preOEC30 was shown to be synthesized on the free ribosomes in the cytoplasm. As there was a possibility that one of the rate-limiting steps in the maturation of the protein may be the translocation of the precursor across the envelope, the contact sites, which have been suggested to be the sites of translocation across the envelope membranes of precursor proteins, were observed by election microscopy. From electron microscopy of the envelope membranes, it was suggested that the outermost membrane of the chloroplast envelope is a significant barrier for the translocation of the precursors, or that the small amount of the contact sites formed by all the three membranes is a rate-limiting step for the process of import of precursor proteins in chloroplasts. To clarify the possible relationship between the

structure of precursors and the pathway for their transport to chloroplasts in *Euglena*, a cDNA clone for OEC30 was isolated and characterized. The high degree of homology was indicated between the mature protein sequences of *Euglena* OEC30 and higher plants and *Chlamydomonas* OEC33. However, the degree of homology between the presequences of *Euglena* preOEC30 and higher plants preOEC33 was shown to be considerably low. The presequence of the precursor of OEC30 was shown to include two hydrophobic domains. The C-terminal hydrophobic domain corresponded to the thylakoid transfer domain, which directs the protein to the lumen of thylakoids, while the N-terminal hydrophobic domain had a signal peptide-like structure, and no homology was identified between this hydrophobic domain and the amino acid sequences of the chloroplast transit peptides of higher plants.

In chapter III, the author examined whether the infomation in the transit peptide of *Euglena* preOEC30 was recognized by the protein transport apparatus in chloroplasts of higher plants. Although the efficiency of transport was lower than that of pea preOEC33, the precursor of *Euglena* preOEC30 was found to be imported into spinach chloroplasts and to be translocated to the lumen as the mature form. The transit peptide of the precursor was further characterized using the chimeric precursor constructed from *Euglena* preOEC30 and pea preOEC33. The whole transit peptide of *Euglena* preOEC30 was competent to direct the protein to spinach chloroplasts but was insufficient for transport the protein across the envelope membranes. The N-terminal hydrophobic domain itself was shown not to possess the capability to direct to spinach chloroplasts. It was suggested that the suitable combination of transit peptides and mature proteins was likely to be necessary for transport of proteins into higher plants chloroplasts. From the import experiments with PE-E chimera, the putative thylakoid transfer domain of *Euglena* preOEC30 was shown to possess the structural and functional similarity to the thylakoid transfer domain of higher plants. There is a possibility that the thylakoid

transfer apparatus in the thylakoid membrane may be similar between *Euglena* and higher plants chloroplasts.

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REFERENCES

Arnon, D. I. (1949) *Plant Physiol.* 24, 1-15.

Bartlett, S. G., Grossman, A. R., and Chua, N.-H. (1982) in *Methods in Chloroplast Molecular Biology* (Edelman, M., Hallick, R. B., and Chua, N.-H, eds.) pp. 1081-1091, Elsevier Biomedical Press, Amsterdam.

Bassham, D. C., Bartling, D., Mould, R. M., Dunbar, B., Weisbeek, P., Hermann, R. G. and Robinson, C. (1991) *J. Biol. Chem.* 266, 23606-23610.

Bauerle, C., Dorl J. and Keegstra K. (1991) *J. Biol. Chem.* 266, 5884-5890.

Chan, R. L., Keller M, Canaday, J, Weil, J-H, and Imbault, P. (1990) *EMBO J.* **9**, 333-338.

Clausmeyer, S., Klösgen, R. B. and Herrmann, R. G. (1993) *J. Biol. Chem.*, 268, 13869-13876.

Cline, K., Werner-Washburne, M., Andrews, J., and Keegstra, K. (1984) *Plant Physiol.* 75, 675-678.

Cline, K., Werner-Washburne, M., Lubben, T. H., and Keegstra, K. (1985) *J. Biol. Chem.* 260, 3691-3696.

de Boer, A. D., and Weisbeek, P. J. (1991) *Biochim. Biophys. Acta* 1071, 221-253.

Douglas, M. G., McCammon, M. T. and Vassarotti, A. (1986) *Microbiol. Rev.* **50**, 166-178.

Douglas, S. E. (1992) *FEBS Lett.* 298, 93-96.

Ehara, T., Sumida, S., Osafune, T., and Hase, E. (1984) *Plant Cell Physiol.* **25**, 1133-1146.

Ellis R. J. (1981) *Annu. Rev. Plant Physiol.* **32**, 111-137.

Flachmann, R., Michalowski, C. B., Löffelhardt, W. and Bohnert, H. J. (1993) *J. Biol. Chem.* **268**, 7514-7519.

Gavel, Y., and von Heijne, G. (1990) *FEBS Lett.* **261**, 455-458.

Gibbs, S. P. (1970) *Ann. NY Acad. Sci.* **175**, 454-473.

Gibbs, S. P. (1981) *Ann. NY Acad. Sci.* **361**, 193-207.

Gumpel, N. J. and Smith, A. G. (1992) *Eur. J. Biochem.* **210**, 721-727.

Hara-Nishimura, I., Matsumoto, T., Mori, H., Nishimura, M., Hara, R. and Hara T. (1990) *FEBS Lett.* **271**, 106-110.

Hageman, J., Robinson, C., Smeekens, S. and Weisbeek, P. (1986) *Nature* **324**, 567-569.

Hageman, J., Baecke, C., Ebskamp, M., Pilon, R., Smeekens, S. and Weisbeek, P. (1990) *The Plant Cell* **2**, 479-494.

Halpin, C., Elderfield, P. D., James, H. E., Zimmermann, R., Dunbar, B. and Robinson, C. (1989) *EMBO J.*, **8**, 3917-3921.

Hiramatsu, H., Mizobuchi, A., Mori, H. and Yamamoto, Y. (1991) *Plant Cell Physiol.* **32**, 881-889.

Horrum, M. A. and Schwartzbach, S. D. (1980) *Planta* **149**, 376-383.

Houlné, G., and Schantz, R. (1988) *Mol. Gen. Genet.* **213**, 479-486.

James, H. E., Bartling, D., Musbrove, J. E., Kirwin, P. M., Herrmann, R.G. and Robinson, C. (1989) *J. Biol. Chem.* **264**, 19573-19576.

Keegstra, K. (1989) *Cell* **56**, 247-253.

Kishore, R., Muchhal, U. S. and Schwartzbach, S. D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11845-11849

Knott, T. G. and Robinson, C. (1994) *J. Biol. Chem.* **269**, 7843-7846.

Ko, K. and Cashmore, A. R. (1989) *EMBO J.* **8**, 3187-3194.

Ko, K., Granell, A., Bennett, J. and Cashmore, A. R. (1990) *Plant Mol. Biol.* **14**, 217-227.

Kuwabara, T., Reddy, K. J. and Sherman, L. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8230-8234.

Kyte, J. and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105-132.

Laemmli, U. K. (1970) *Nature* **227**, 680-685.

Larkins, B. A. and Davies, E. (1975) *Plant Physiol.* **55**, 749-756.

Larkins, B. A., Bracker, C. E. and Tsai, C. Y. (1976) *Plant Physiol.* **57**, 740-745.

Laskey, R. A. and Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335-341.

Lefort-Tran, M., Pouphile, M., Freyssinet, G. and Pineau, B. (1980) *J. Ultrast. Res.* **73**, 44-63.

Lubben, T. H. and Keegstra, K. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5502-5506.

Mayfield, S. P., Rahire, M., Frank, G., Zuber, H. and Rochaix, J.-D. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 749-753.

Meadows, J. W., Shackleton, J. B., Hulford, A. and Robinson, C. (1989) *FEBS Lett.* 253, 244-246.

Mizobuchi, A. and Yamamoto,Y.(1989) *Biochim. Biophys. Acta* **977**, 26-32.

Monroy, A. F., McCarthy, S. A. and Schwartzbach, S. D. (1987) *Plant Sci.* **51**, 61-76.

Mori, H., Takeda-Yoshikawa, Y., Hara-Nishimura, I. and Nishimura, M. (1991) *Eur. J. Biochem.* **197**, 331-336.

Olsen, L. J., Theg, S. M., Selman, B. R. and Keegstra, K. (1989) *J. Biol.Chem.* **264**, 6724-6729.

Olsen, L. J. and Keegstra, K. (1992) *J. Biol.Chem.* **267**, 433-439.

Osafune, T, Schiff, J. A.,and Hase, E. (1991) *Exp. Cell Res.* **193**, 320-330.

Ostrem, J. A., Ramage, R. T., Bohnert, H. J., and Wasmann, C. C. (1989) *J. Biol. Chem.* 264, 3662-3665.

Pain, D., Kanwar, Y. S. and Blobel. G. (1988) *Nature* 331, 232-237.

Perbal, B. (1988) in *A Practical Guide to Molecular Cloning*, pp. 789-792. John Wiley, New York.

Pfanner, N., Hartl, F.-U. and Neupert, W. (1988) *Eur. J. Biochem.* **175**, 205-212.

Rikin, A, and Schwartzbach, S. D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5117-5121.

Rochaix, J. D. and Erickson, J. (1988) *Trends Biochem. Sci.* **13**, 56-59.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) *Science* **239**, 487-491.

Saitou, N. and Nei, M. (1987) *Mol. Biol. Evol.* **4**, 406-425.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) in *Molecular Cloning : A Laboratory Manual, 2nd ed.* Cold Spring Harbor Laboratory, Cold Spring Harbor.

Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H., Roe, B. A. (1980) *J. Mol. Biol.* **143**, 161-178.

Scaramuzzi, C. D., Hiller, R. G. and Stokes, H. W. (1992) *Curr. Genet.* **22**, 421-427.

Schiff, J. A., Schwartzbach, S. D., Osafune, T. and Hase, E. (1991) *J. Photochem. Photobiol. B* **11**, 219-236.

Schmidt, G. W. and Mishkind, M. L. (1986) *Ann. Rev. Biochem.* **55**, 879-912.

Schreier, P. H., Seftor, E. A., Schell, J. and Bohnert, H. J. (1985) *EMBO J.* **4**, 25-32.

Schuler, M. A. and Zielinski, R. E. (1989) in *Methods in Plant Molecular Biology,* pp. 89-96. Academic Press, London.

Shackleton, J. B. and Robinson, C. (1991) *J. Biol. Chem.* 266, 12152-12156.

Sharif, A. L., Smith, A. G. and Abell, C. (1989) *Eur. J. Biochem.* **184**, 353-360.

Shashidhara, L. S. and Smith, A. G. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 91-100.

Shashidhara, L. S., Lim, S. H. Shackleton, J. B., Robinson, C. and Smith, A. G. (1992) *J. Biol. Chem.* **267**, 12885-12891.

Smeekens, S., Bauerle, C., Hageman, J., Keegstra, K., and Weisbeek, P. (1986) *Cell* 46, 365-375.

Smeekens, S., Bauerle, C., Hageman, J., Keegstra, K. and Weisbeek, P. (1986) *Cell* **46**, 365-375.

Smeekens, S., van Steeg, H., Bauerle, C., Bettenbroeck, H., Keegstra, K. and Weisbeek, P. (1987) *Plant Mol. Biol.* **9**, 377-388.

Smeekens, S. and Weisbeek, P. (1988) *Photosynth. Res.* **16**, 177-186.

Tyagi, A, Hermans, J., Steppuhn, J., Jansson, C., Vater, F. and Hermann, R. G. (1987) *Mol. Gen. Genet.* **207**, 288-293.

Valentin, K. (1993) *Mol. Gen. Genet.* **236**, 245-250.

van den Broeck, G., Timko, M. P., Kausch, A. P., Cashmore, A. R., van Montagu, M. and Herrera-Estrella, L. (1985) *Nature* **313**, 358-363.

von Heijne, G. (1985) *J. Mol. Biol.* **184**, 99-105.

von Heijne, G. (1986) *Nucleic Acids Res.* **14**, 4683-4691.

von Heijne, G. (1988) *Biochim. Biophys. Acta* **947**, 307-333.

von Heijne, G., Steppuhn, J. and Hermann, R. G. (1989) *Eur. J. Biochem.* 180, 535-45.

Wales, R, Newman, B. J., Pappin, D., and Gray, J. C. (1989) *Plant Mol. Biol.* **12**, 439-451

Wasmann, C. C., Reiss, B., Bartlett, S. G. and Bohnert, H. J. (1986) *Mol. Gen. Genet.* **305**, 446-453.

Wasmann, C. C., Reiss, B., and Bohnert, H. J. (1988) *J. Biol. Chem.* 263, 617-619.

Watanabe, A., Minami, E., Murase, M., Shinohara, K., Kuwabara, T. and Murata, N. (1987) in *Progress in Photosynthesis Research,* vol 4, pp. 629-636.

Watson, M. E. E. (1984) *Nucl. Acids Res.* **12**, 5145-5159.

Yamamoto, Y, Doi, M, Tamura, N. and Nishimura, M. (1981) *FEBS Lett.* **133**, 265-268.

Yamamoto, Y. and Nishimura, M. (1983) in *The Oxygen Evolving System of Photosynthesis,* pp. 229-238. Academic Press, Tokyo.

Yamamoto, Y. (1988) *J. Biol. Chem.* **263**, 497-500.

Yamamoto, Y. (1988) *Biochim. Biophys. Acta* **933**, 165-171.

Yamamoto, Y. (1989) *Bot. Mag. Tokyo* **102**, 565-582.

a) Inagaki, J. and Yamamoto, Y. (1992) in *Research in Photosynthesis* (N. Murata ed.), Kluwer Academic Publishers., vol.3, pp.177-180.

b) Kuroda, I., Inagaki, J. and Yamamoto, Y. (1993) *Plant Mol. Biol.* **21**, 171-176.

c) Shigemori, Y., Inagaki, J., Mori, H., Nishinura, M., Takahashi, S. and Yamamoto, Y. (1994) *Plant Mol. Biol.* **24**, 209-215.

d) Inagaki, J., Kuyama, A., Shigemori, Y., Fujita, Y., Hase, T. and Yamamoto, Y. (in preparation).