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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation

Application of precursor proteins with steric hindrance for blocking the process of protein import into chloroplasts *in vitro*
（立体障害を有する前駆体蛋白質が葉緑体への蛋白質輸送に及ぼす影響）

学位論文要約：
Dissertation Summary

Chloroplasts harboring green coloured chlorophyll pigment are the vital cell organelles and are prevalent in photosynthetic eukaryotes. Apart from their characteristic function, in which it converts light energy into chemical energy through the process of photosynthesis, it is also a home for many other important metabolic processes. Most of the chloroplastic genes were transferred to the nuclear genome during the course of evolution; under these circumstances, where more than 95 % of the chloroplastic proteins were synthesized on the cytosolic ribosomes, a proper targeting mechanism to chloroplasts as well as translocation machinery has been evolved in the photosynthetic cells. Much knowledge regarding the entire process of protein import into chloroplasts is still unclear and need to be explored soon by considering the importance of chloroplast on which whole life on earth depends.

Most of the nuclear encoded chloroplastic proteins are synthesized as precursors in the cytosol with their N-terminal transit peptide and are imported into the chloroplasts through protein translocation machinery (translocons) embedded in the outer and inner envelope membranes (Figure 1). Protein import process of these proteins is mainly divided into two stages, “docking” and “translocation”, depending on an energy requirement *in vitro* (Figure 2). In translocation, precursor proteins fully translocate through the double membrane to reach to the interior of chloroplasts, in the presence of a high level of ATP (> 1 mM) at a higher temperature (Figure 2). In contrast, in docking,

precursor proteins irreversibly bind to the chloroplastic surface to form early protein translocation intermediates (PTIs) under limited energy conditions (Figure 2). Precursor proteins are expected to interact sequentially with the translocon components, however regardless of how PTIs were formed during the early stage of protein import under different conditions, addition of a high level of ATP at a higher temperature freed precursors from early PTIs, which results in the completion of translocation. Our current knowledge of chloroplastic translocon is based mainly on the biochemical analyses of the early PTIs formed under docking conditions. However, once precursors depart from the early PTIs, it is impossible to pause the movement of precursors until translocation is completed. Therefore, molecular mechanisms of post-docking stage have not been well-understood. If we are able to manage the movement of translocating precursor by trapping precursors carrying steric hindrance at their carboxyl termini in the translocon to form PTIs under translocation conditions, then we may find a way to solve this subject (Figure 3).

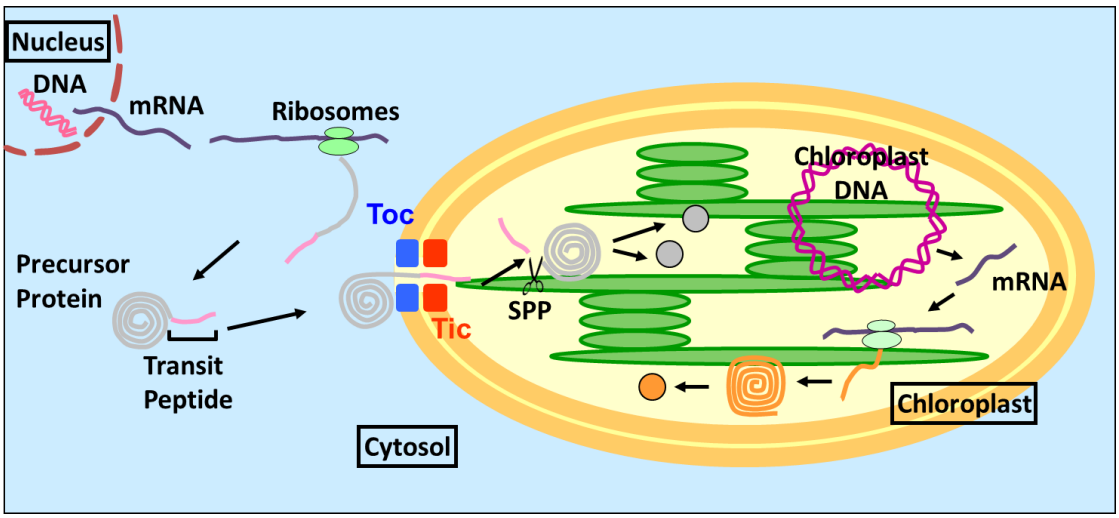

Figure 1: Overview of protein import into chloroplasts.

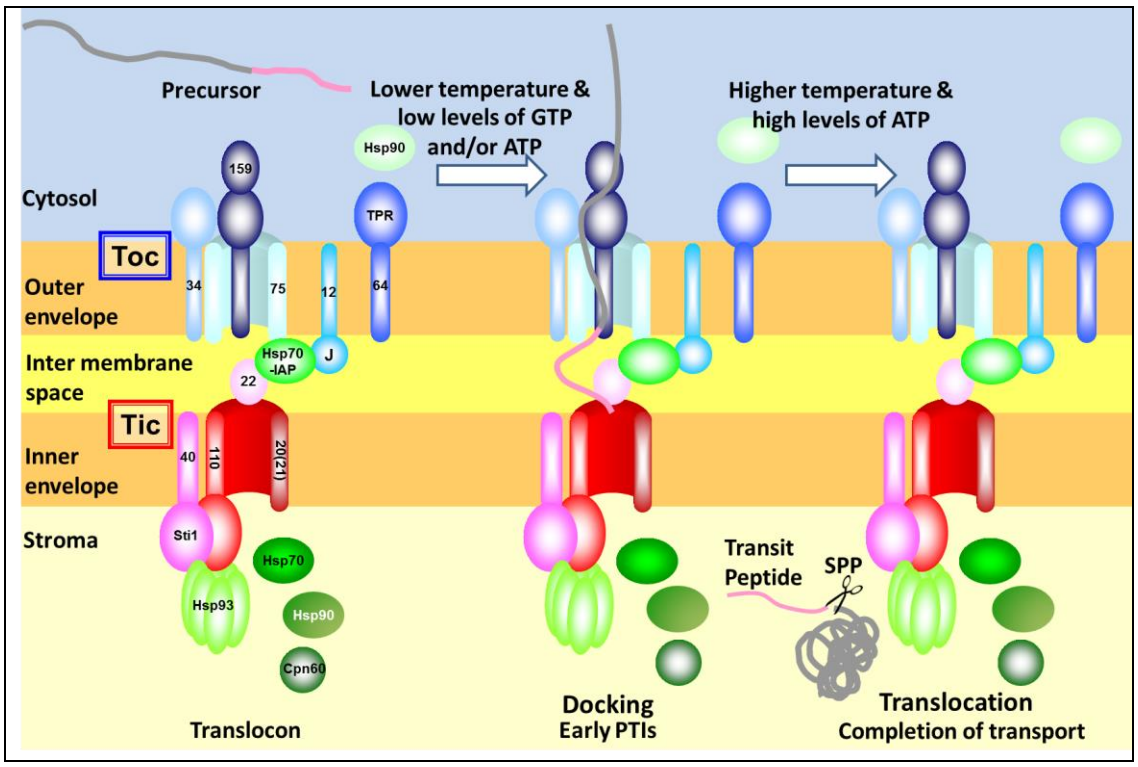

Figure 2: Energy requirements during the process of protein import into chloroplasts.

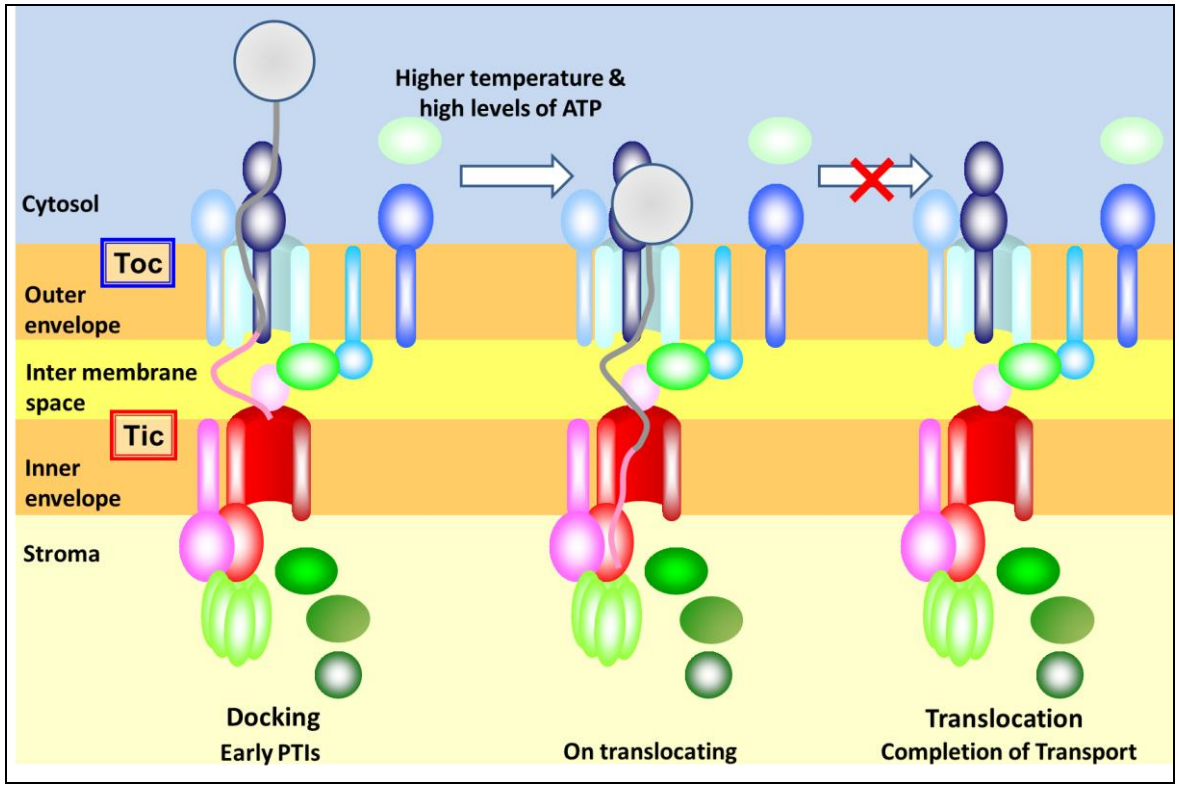

Figure 3: Strategy to plug the translocation channel during the translocation stages of import.

Mitochondria also has double-membrane system as well as chloroplasts, many mitochondrial proteins are also nuclear-encoded, so that these proteins should be imported into mitochondria. In case of mitochondrial protein import, nuclear encoded mitochondrial proteins are synthesized on the cytosolic ribosomes as precursors and are imported into mitochondria through multi subunit protein complexes located in the outer and the folded inner membrane by using the driving force of an electrochemical gradient present across the inner membrane. In mitochondria, the precursor protein fused to mouse dihydrofolate reductase (DHFR) has blocked import once DHFR structure was stabilized in the presence of methotrexate (MTX) (Eilers and Schatz, 1986). By analyzing the trapped precursor, progress of research on mitochondrial protein import was dramatically enhanced. To this end, two strategies which could be employed for blocking the process of protein import into chloroplasts mainly includes, developing a precursor protein which is fused to the tightly folded domain and post translational attachment of the folded structure to the precursor protein.

Firstly, we have employed a precursor which is fused to tightly folded domain. The similar kind of strategy has already been applied on chloroplastic protein import. However, protein import into chloroplasts was not blocked by the tightly folded precursors fused with mouse cytosolic DHFR in the presence of MTX (America *et al*, 1994; Endo *et al*, 1994). In the current investigation, we made an attempt to reevaluate whether DHFR—MTX interaction did not significantly affect protein import into chloroplasts. Precursors fused to DHFR from mouse reported previously was cell-free synthesized incorporating radio-active amino acids without purification, indicating that the amount of precursors were limited and synthesized precursors were contaminated with various cellular factors and small molecules, which might affect blockage of protein import. By applying recombinant precursors expressed in *E. coli* cells, we had developed *in vitro* chloroplastic protein import assay system (Inoue *et*

al, 2008). Important features of this assay system are: it can handle the large amount of precursor, import reaction is analyzed without exogenous factors, thus manageable by strict energy conditions. But all of *E. coli* overexpressed precursors prepared so far, were recovered in the inclusion bodies and had to be solubilized in 8 M urea prior to the import reaction. However, denaturation step should be avoided, if the precursor with a tightly folded domain was applied for plugging translocation channel. Therefore, we designed a precursor protein which was assembled with soluble components. Since DHFR is tightly folded in the presence of MTX, in addition, to avoid the use of a gene from heterologous origin, DHFR from *E. coli* (*Ec*DHFR) was selected to be a major component of the mature part of the precursor. For a detection and purification purpose, human influenza hemagglutinin protein (HA) epitope tag, biotin acceptor peptide (BAP), and histidine affinity tag (HAT) were chosen. HAT, instead of hexahistidine (H6) tag, was picked because it was considered to be more soluble than artificial H6 tag. In addition, to extend the distance between the transit peptide and *Ec*DHFR, one unit of the random coil linker (r1) consisted with the quintuple penta-peptides of GGGGS ($(G_4S)_5$) was inserted to complete the precursor construction. Our newly prepared precursor protein was recovered in a soluble form in *E. coli* cells after overexpression and has retained chloroplast import competency (Pohare and Akita, 2016). DHFR portion of this precursor was tightly folded in the presence of MTX and import of MTX bound precursor was inhibited, though not completely. In addition, after fractionation of chloroplasts, processed precursor protein was recovered in the soluble fraction (Akita and Pohare, 2016). These results indicate the presence of unfolding activity within chloroplasts, which allow *Ec*DHFR-fused precursor to be translocated through envelope membranes.

Secondly, for the post translational attachment of the folded structure, biotin-streptavidin (SA) technology was utilized. Interaction between biotin and SA is known to be one of the strongest

non-covalent interactions in nature. SA is a homo-tetramer, thus binds up to four biotin molecules, that more than one biotinylated precursor may be bound to SA. Howarth *et al* (2006) developed monovalent SA consisted of one subunit of wild-type and three mutant subunits deficient in biotin binding. Unlike monomeric SA, monovalent SA retains the same level of biotin affinity and thermostability as the wild-type SA. Monovalent SA was associated with the precursor protein carrying BAP at its carboxyl terminal overexpressed in *Escherichia coli*. When this precursor-monovalent SA complex was applied for *in vitro* chloroplastic import assay, translocation of precursor was not blocked. Further, we developed a more stable form of monovalent SA; cross-linked monovalent traptavidin which has 10-fold slower biotin dissociation as compared to the monovalent SA and has enhanced stability due to insertion of a covalent cross-link across dimer-dimer interface. Although the import of biotinylated precursors was inhibited in the beginning of the reaction, but even cross-linked monovalent traptavidin does not prevent the biotinylated precursors from being imported into chloroplasts. In this case too, the processed precursor protein was recovered in the soluble fraction after fractionation of chloroplasts and remained protected from the thermolysin digestion. This suggested the presence of strong denaturing activity at the chloroplastic surface or the strong pulling force enough to dissociate monovalent SA or cross-linked monovalent traptavidin from biotinylated precursor. If in the latter case, apart from the interest in protein targeting mechanism, to investigate such strong motor, if presents, is valuable for innovating a biological nanomotor.

During the course of precursor preparations, gene manipulation was essential. When we employed gene manipulation, we found a rapid and simple, recombination based cloning method in *E. coli*. Various types of enzyme kits serving the purpose of cloning have already been commercialized and are available from several sources. When cloning with such kits, control experiments in which the

enzyme mixture was omitted often also produced transformants that carried the desired recombinant plasmids. Such an undesirable outcome served as an impetus for designing a cloning technique. Here, we report a time-saving, cost-effective, and simple cloning technique that was serendipitously discovered. After optimizing the experimental conditions, such as the molar ratio of two fragments and the length of overlaps between the ends of two fragments, we found that the desired transformants may be obtained when a mixture of two DNA fragments containing at least 12 bp of identical nucleotide sequences at their terminal ends is introduced into *E. coli* cells (Pohare and Akita, 2017). To mechanistically explain our cloning procedure, double-stranded DNA specific exonucleases, such as RecE, are present in *E. coli* cells, once a vector and an insert fragment are introduced in cells after heat shock treatment, single-stranded DNA overhangs will be created at both ends of the fragments. Single stranded overhangs favor annealing of these fragments *in vivo* and the gap is filled in by a polymerase, followed by being sealed by a ligase. Thus, we propose a mechanism which operates *in vivo* and functions similarly to that of the SLiCE method (Zhang *et al*, 2012). Because this technique is based on homologous recombination *in vivo*, cloning is independent of restriction endonuclease recognition sites at the cloning site, and a gene of interest can thus be cloned into plasmid at the desired site without additional nucleotide sequences. Therefore, most of the time-consuming steps required in classical cloning protocols are avoided.

References

Akita, M., Pohare, M.B. Chloroplastic protein import characteristics of dihydrofolate reductase (DHFR) fused recombinant precursor protein in the presence of methotrexate. *Biosci., Biotechnol. Res. Asia*, 2016; 13(4): 2351–8.

America, T., Hageman, J., Guera, A., Rook, F., Archer, K., Keegstra, K., Weisbeek, P. Methotrexate does not block import of a DHFR fusion protein into chloroplasts. *Plant Mol. Biol*., 1994; 24: 283–94.

Eilers, M., Schatz, G. Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature*, 1986; 322: 228–32.

Endo, T., Kawakami, M., Goto, A., America, T., Weisbeek, P., Nakai, M. Chloroplast protein import. Chloroplast envelopes and thylakoids have different abilities to unfold proteins. *Eur. J. Biochem.*, 1994; 225: 403–9.

Howarth, M., Chinnapen, D.J., Gerrow, K., Dorrestein, P.C., Grandy, M.R., Kelleher, N.L., El-Husseini, A., Ting, A.Y. A monovalent streptavidin with a single femtomolar biotin binding site. *Nat. Methods*, 2006; 3(4): 267–73.

Inoue, H., Ratnayake, R.M.U., Nonami, H., Akita, M. Development and optimization of an *in vitro* chloroplastic protein import assay using recombinant proteins. *Plant Physiol. Biochem*., 2008; 46: 541–9.

Pohare, M.B., Akita, M. A rapid and simple, recombination based cloning method in *Escherichia coli*. *Biosci., Biotechnol. Res. Asia*, 2017; 14(1): (In press).

Pohare, M.B., Akita, M. Development of a precursor in a soluble form for protein import into chloroplasts. *International Journal of Bio-Technology and Research*, 2016; 6(5): 9–18.

Zhang Y., Werling U., Edelmann W., SLiCE: a novel bacterial cell extract-based DNA cloning method. *Nucleic Acids Res*., 2012; 40(8): e55.