12-2003

Involvement of Transit Peptide Aromatic Residues in Precursor Interaction(s) with the Chloroplast Import Apparatus

Huixia Zhang
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Huixia Zhang entitled "Involvement of Transit Peptide Aromatic Residues in Precursor Interaction(s) with the Chloroplast Import Apparatus." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Barry D. Bruce, Major Professor

We have read this thesis and recommend its acceptance:

Bruce D. McKee Albrecht von Arnim

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Huixia Zhang
December 2003

DEDICATION

This thesis is dedicated to to my parents

Yulan Cui and Xi’en Zhang

and

my dear husband

Pu Liu

Acknowledgements

First of all, I would like to thank my research advisor Dr. Barry D. Bruce. Thanks him for giving me this opportunity to work in his lab and for the support and training I received during the two years. It has been a great pleasure to work under his supervision with his unlimited enthusiasm toward science. I am also very grateful to my other committee members, Dr. Bruce McKee and Dr. Albrecht von Arnim for their guidance and support in thesis writing.

I am thankful to the present and past members of Bruce lab, particularly Sarah Wright, Dr. Carole Dabney-Smith, Dr. Chitra Subramanian, David McWilliams, Anton Mitsky, Evan Reddick, Michael Vaughn, for their help. I would remember those bad English they taught me forever. It is them who brought enjoyment to the lab.

I am also grateful to those wonderful friends I met here in Knoxville, Tennessee. It is their companion that helped me cure homesickness, loneliness and enjoy the life here. Wish them good luck in their graduate study and life.

I am grateful to my parents and brothers for their constant love and support while I am pursuing my goal. It is their expectation that drives me going forward all these years. Without them, I could not accomplish what I have finished.

Finally, I would thank my husband, Pu Liu, for his love, and support since we met.

Abstract

The import of nuclear-encoded preproteins into chloroplast is mediated by the transit peptide located in the N-terminus of the preproteins. A semi-conserved FGLK motif is identified in most of the transit peptides bioinformatically, despite the lack of homology in the primary sequence. To investigate the role of phenylalanines in the FGLK motifs of SStp in the interaction with outer envelope import apparatus, mutations were made on the two motifs found in prSSU from tobacco: F_{26}TGLK and F_{35}PVSRK. These mutations include F→W, F→S, and F→A in both of the motifs. The WT precursor (prSSU), 6 single prSSU mutants, 9 double prSSU mutants and mature protein were expressed and purified from *E. coli.* The activities of 13 out of 15 mutant proteins are then tested as competivie inhibitors of the import of ^{35}S-prSSU. The increase in IC_{50} indicated that both phenylalanines at position 26th and 35th are important for the import process. Membrane disruption assay showed that all these mutants have slightly reduced membrane activity compared to wild type, suggesting that interaction with membrane lipid components is secondary to other interactions with proteinaceous component in the early event of the import process. Tryptophan fluorescence assay using the transit peptide mutants F26W-SStp and F35W-SStp demonstrated that the two loosely conserved FGLK motifs have different positions when exposed to a hydrophobic environment, indicative of different roles they might have in the recognition/binding process.

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Chapter 1 Introduction

Like all eukaryotes, plant cells have membranes, ribosomes, a nucleus, Golgi apparatus, endoplasmic reticulum (ER), and mitochondria. The two differences between plant cells and animal cells are that plant cells have an additional organelle, named the plastid, and a rigid cell wall made of cellulose. The chloroplast is one member of the plastid family that arose as a result of an endosymbiotic event, wherein a eukaryotic ancestral cell engulfed a photosynthetic cyanobacteria (Weeden 1981), thus giving rise to the double outer membrane of each member of the plastid family. During evolution, most of the cyanobacterial genes were transferred to the host's nucleus (Kaneko et al 1996), so a large majority of chloroplast proteins are synthesized on the free ribosomes of the cytoplasm and are transported back into the organelle post-translationally. This is accomplished with the assistance from the translocons located at the outer and inner envelope membranes of chloroplast, Toc (**T**ranslocator in the **o**uter envelope membrane of **c**hloroplast) and Tic (**T**ranslocator in the **i**nner envelope membrane of **c**hloroplast) (Schnell et al 1997; Keegstra & Cline 1999; Fuks & Schnell 1997; Heins et al 1998; Soll 2002; Schnell & Hebet 2003). These gene products are redirected to their "ancestral" compartment via the acquisition of a transit peptide, which is located in the N-terminus of the precursor coding sequence and has been shown to be both necessary and sufficient to ensure

high fidelity transport back into the organelle (Anderson & Smith 1986; Mishkind et al 1985; Van den Broeck et al 1985).

1.1 Protein import into chloroplasts

A large part of the genetic information of the bacteria (cyanobacteria) was transferred to the eukaryotic host during endosymbiosis (Kaneko et al 1996). As a result, most chloroplast proteins that are required for structure and function are encoded by the nuclear genome and are synthesized on the free cytoplasmic 80S ribosomes (Abdallah et al 2000; Cavalier-Smith, 2000). Sequence analysis of the Arabidopsis genome suggests that potentially 3,574 proteins are synthesized as precursor proteins and targeted back into the chloroplast (Initiative 2000) and only 87 proteins (Sato et al 1999; Abdallah et al 2000) are encoded locally in the plastid. Those proteins that are synthesized in the cytoplasm are called precursors, and these precursors are encoded as higher molecular weight proteins with an N-terminal cleavable targeting sequence called the transit peptide, which is essential for the post-translational uptake of precursors by the chloroplast.

During transport, these precursor proteins usually remain partly unfolded. This process is assisted by the transport apparatus located on the two-layer membrane of the chloroplast, Toc and Tic. The main subunits found in Toc from pea chloroplasts are Toc159/86 (Hirsch et al 1994; Kessler et al 1994), Toc34 (Kessler et al 1994; Seedorf et al 1995), Toc75 (Schnell et al 1994; Tranel et al 1995) and Toc64 (Sohrt &

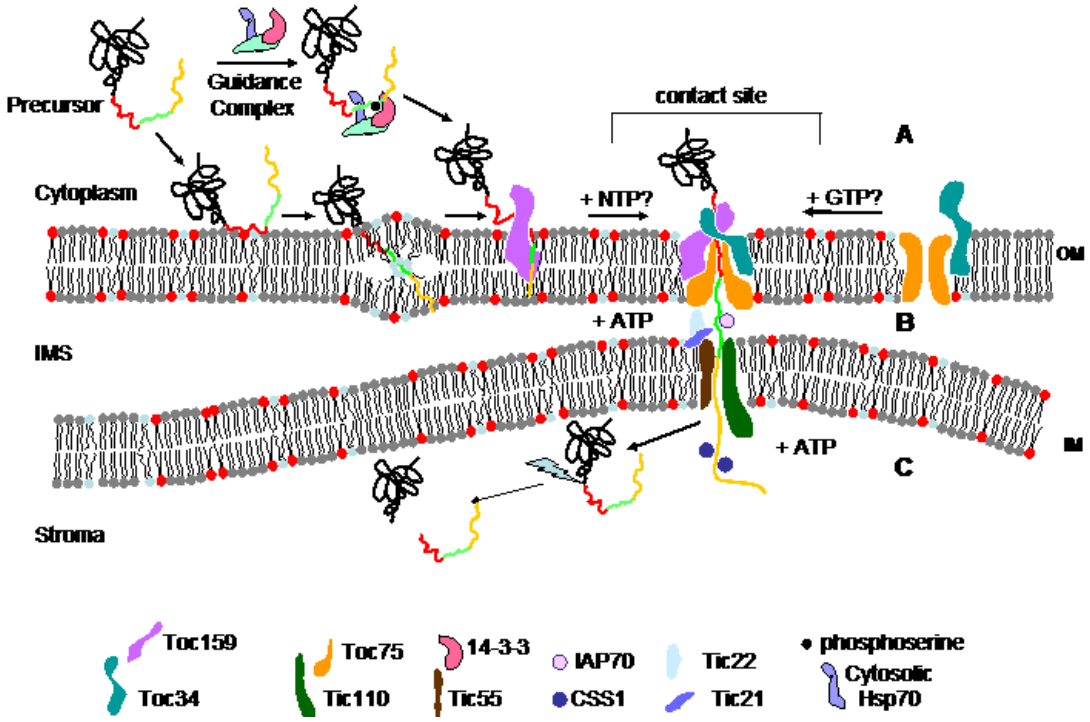
Soll, 2000). The main Tic members are Tic22 (Kouranov et al 1998), Tic20 (Kouranov et al 1998; Kouranov & Schnell 1997), Tic40 (Wu et al 1994; Ko et al 1995; Stahl et al 1999) and Tic110 (Kessler et al 1996; Lübeck et al 1996).

1.1.1 General import pathway

Precursor proteins are synthesized on the free 80S ribosomes in the cytosol with the N-terminal cleavable extension called the transit peptide that targets the precursor to the organelle. This back home journey is a complicated process that can be divided into 3 stages according to energy requirements (Figure 1-1). The first stage (stage A) is the initial binding of a preprotein by receptors/lipids in the outer envelope membrane, which is energy-independent and is a reversible process (Ma et al 1996). At this stage, the transit peptide associates with the receptor(s) with low affinity in the absence of nucleoside triphosphate.

There are four possible competing pathways for trafficking of preproteins from the cytoplasm to the Toc apparatus. First, following phosphorylation of a specific serine of the transit peptide by a cytosolic kinase in the cytoplasm (Waegemann & Soll 1996), the preprotein is recognized by a guidance complex composed of a 14-3-3 dimer and a molecular chaperone Hsp70 (May & Soll 2000). This guidance complex then delivers the preprotein to the receptors on the outer membrane (May & Soll 2000). Alternatively, the preprotein binds to the soluble form of Toc159 in the cytosol, this complex [Toc159: preprotein] then docks at the outer envelope membrane and

Figure 1-1 General import pathway for chloroplast preproteins synthesized in the cytoplasm. Preprotein is synthesized in the cytoplasm with a transit peptide. The transit peptide may be phosphorylated and is bound by a guidance complex. A: Initial binding of preprotein to Toc complex; B: Formation of import intermediate requiring ATP (< 100 μM); C: Complete translocation of preprotein across the membrane and final arrival at the stroma in the presence of ATP (1-3 mM).


Precursor
Guidance Complex
contact site
A
Cytoplasm
+ NTP?
+ GTP?
OM
+ ATP
B
IMS
IM
+ ATP
C
Stroma
Toc159
Toc34
Toc75
Tic110
14-3-3
Tic55
IAP70
CSS1
Tic22
Tic21
phosphoserine
Cytosolic Hsp70

somehow hands the preprotein over to other subunit(s) of the translocon (Hiltbrunner et al 2001). Third, preproteins may bind directly with the outer envelope membrane, through specific interactions with chloroplast specific lipids: MGDG (monogalactosyldiacylglycerol), DGDG (digalactosyldiacylglycerol), and SL (sulfolipid). After lipid interaction, there may be a reciprocal change in both transit peptide structure and the lipid organization of the envelope. Then the membrane-associated transit peptide gets recognized and bound by the outer membrane receptors (Bruce 2000). Finally, the preproteins may interact directly with the Toc translocon in the outer membrane without any other source of interaction. Which of these four possibilities is utilized will depend upon the properties of a given precursor, as well as the developmental and physiological status of the plant cell and its plastid.

In the second stage (stage B), low concentrations of ATP (<100 μM) and GTP are hydrolyzed in the cytoplasm or intermembrane space, resulting in an irreversible association of precursor proteins with the translocation machinery of both envelope membranes (Olesen & Keegstra 1992). Crossing linking experiments have shown that the preprotein is partially inserted across the outer membrane and is in contact with the components in the inner membrane envelope (Akita et al 1997; Nielsen et al 1997; Kouranov et al 1997). The precursor at this stage is still outside of the chloroplast and is sensitive to exogenous protease (Olsen & Keegstra 1992). This is considered a translocation intermediate (Ma et al 1996; Nielsen et al 1997).

The last step (stage C) is the complete translocation of the proteins through the two layer membranes and their final arrival in the stroma. This step requires high levels of ATP (1-3 mM) within the stroma (Theg et al 1989; Olsen & Keegstra 1992). The ATP hydrolysis is

presumably mediated by stromal molecular chaperones, at least one, Hsp93, belonging to the Hsp100 family of molecular chaperones, was found to interact with the import complex (Akita et al 1997; Nielsen et al 1997). As the precursor enters the chloroplast, the transit peptide is cleaved off by the stromal processing peptidase (SPP) and the mature protein begins the process of folding and assembly (Oblong & Lamppa 1992; VanderVere et al 1995; Richter & Lamppa 1998).

1.1.2 Toc complex

The translocation of chloroplast preproteins is assisted by the translocon complex located in the outer envelope membrane that is called Toc (**T**ranslocon in the **o**uter envelope membrane of **c**hloroplast). When the preprotein arrives at the outer membrane, a specific recognition is initiated. This recognition is facilitated by the outer membrane receptors and translocation channel. The Toc complex recognizes, binds and translocates precursor proteins across the outer membrane of the chloroplast. There are four major subunits that have been found in this complex from pea, Toc86/159, Toc34, Toc75 and Toc64 (Schnell et al 1997). The associated number indicates the molecular size of each subunit in kilodaltons.

1.1.2.1 Toc86/159

Toc159 is postulated to be the chloroplast protein import receptor in the outer envelope membrane. It was shown to be in closest association with bound preproteins in cross-linking experiments that favored the initial binding prior to translocation in the absence of energy (Perry & Keegstra 1994; Ma et al 1996; Kouranov & Schnell, 1997;

Akita et all 1997). Furthermore, addition of antibodies against Toc159 to isolated chloroplasts blocked precursor binding to the chloroplast surface (Hirsch et al 1994). However, other experiments showed that the initial binding of preprotein to Toc159 can be bypassed in the import process (Chen et al 2000).

Toc159 has a three-domain structure: an N-terminal acidic domain, a central GTP-binding domain and the C-terminal membrane-associated domain (Kessler et al 1994; Hirsch et al 1994; Chen et al 2000). The amino-terminal domain and the central domain are exposed to the cytosol, and in isolated chloroplasts, they are highly sensitive to exogenous protease (Kessler et al 1994; Hirsch et al 1994). The GTP-binding domain of Toc159 does not have much homology to other GTPases except another preprotein receptor in the outer membrane, Toc34 (Kessler et al 1994; Hirsch et al 1994). The net positive feature of the transit peptide and the acidic nature of the N-terminal cytosolic domain of Toc159 might lead to the assumption that an electrostatic interaction is the driving force behind the binding of preproteins to the receptor (Pfanner et al 1997; Lithgow 2000). However, when Toc159 is degrated to Toc86, a proteolytic product lacking the acidic motif, proteins were still found to be imported into the isolated chloroplast, though with reduced import efficiency (Bölter et al 1998). This suggests that electrostatic interaction is not the only force involved in the recognition/binding process.

Three homologues of pea Toc159 (psToc159) were found in Arabidopsis, atToc159, atToc132 and atToc120. These three proteins show high similarity to each other and psToc159, especially in the GTP-binding domain and the membrane anchor domain, except that the amount of transcript product of AtToc159 is five to ten fold higher than those of Toc132 and Toc120 (Bauer et al 2000; Hiltbrunner et al 2001). Analysis of

atToc159-null *Arabidopsis* mutant *ppi2* (plastid protein import), which has a T-DNA insertion in the gene coding for Toc159, found that mutant plants die at the seedling age and proplastids fail to develop into chloroplasts, suggesting the importance of atToc159 in the biogenesis of chloroplasts (Bauer et al 2000). However, protein import can still occur, suggesting another import pathway exists. atToc132 and atToc120 are proposed to maintain the basal protein import in *ppi2,* together with Toc34/Toc33, which is more highly expressed in this mutant compared to wild type.

Another interesting phenomenon regarding atToc159 is that is was found to have both soluble and membrane-bound forms and the integration of atToc159 into the chloroplast outer membrane requires its binding to the cytosolic domain of atToc33 (Hiltbrunner et al 2001; Smith et al 2003). Therefore, another function of atToc159 may be targeting cytosolic preproteins to the chloroplast surface as described above.

1.1.2.2 Toc34

Toc34 was first identified as a GTP-binding protein in the chloroplast import machinery (Kessler et al 1994). The full length protein has a large N-terminal domain exposed to the cytosol which contains the GTP-binding motif, one transmembrane domain and a C-terminal small tail projecting into the intermembrane space (Seedorf et al 1995; Li et al 1997; Sun et al 2002). Toc34 and preproteins were shown in close proximity during import (Kessler et al 1994; Kouranov & Schnell 1997) and also in association with the translocation pore of Toc75 (Seedorf et al 1995). The soluble cytosolic domain of Toc34 lacking the transmembrane domain (Toc34ΔTM) was shown to compete with the import of prSSU into chloroplasts (Schleiff et al 2002). These both supported the function of

Toc34 as a preprotein receptor. Toc34 and Toc159, the two preprotein receptors in the outer membrane, can work cooperatively, since Toc34 was shown to be overexpressed in order to compensate for the loss of Toc159 in *ppi2* mutants (Bauer et al 2000).

The GTP binding motif in the N-terminal domain of Toc34 shows high homology to that of Toc159 and is hypothesized to have a regulatory function during precursor import (Kessler et al 1994; Seedorf et al 1995; Kouranve & Schnell 1997). The GTP-bound form of Toc34 has a higher affinity for preproteins (Sveshnikova et al 2000; Schleiff et al 2002). GTP hydrolysis by Toc34 is highly stimulated by the presence of preproteins and results in GDP-bound Toc34 which shows low affinity for preprotein (Sveshnikova et al 2000; Schleiff et al 2002; Jelic et al 2002). The GTP hydrolysis rate can be further regulated by receptor dimerization (Sun et al 2002). Toc34 can be phosphorylated by a protein kinase associated with the outer membrane (Fulgosi & Soll 2002), which blocks the binding of GTP (Fulgosi & Soll 2002; Sveshnikova et al 2000).

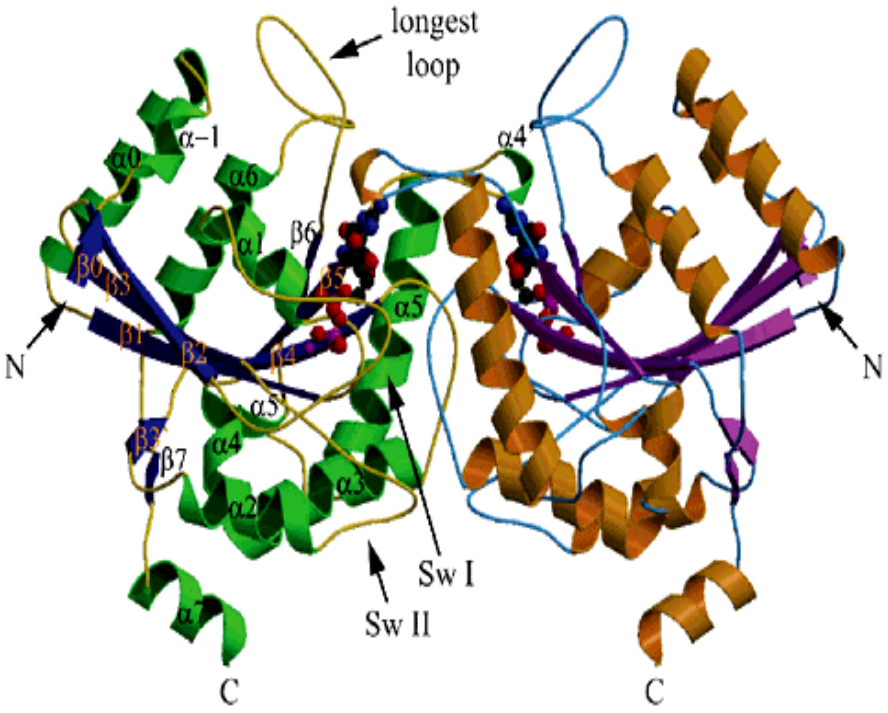
The crystal structure was recently obtained for the cytosolic domain of pea Toc34 in the presence of GDP and Mg^{2+} (Sun et al 2002). In the crystal structure, Toc34 molecules exist as dimers and the ribbon structure of the dimer, which looks like a butterfly, is shown in Figure 1-2. The wings, antennae and body of the butterfly are represented by α-helices and β sheets of the dimer, the two longest loops and the dimer interface, respectively (Sun et al 2002).

Two homologues of pea Toc34 were found in Arabidopsis, atToc33 and atToc34 (Jarvis et al 1998). *atToc33* is preferentially expressed during the early stages of seedling development, whereas *atToc34* is expressed at constitutively low levels during all stages

Figure 1-2 Structure of the GDP-bound form of the Toc34 dimer and its interface.

The two Toc34 molecules are related to one another by a crystallographic two-fold axis. Monomer 1 is colored green, dark blue and yellow; monomer 1' is colored orange, violet and light blue. GDP is represented as a ball-stick model, and Mg^{2+} is shown in magenta. Switches I and II, and the longest loop are labeled.

Adapted from Sun et al, *Nature Struct. Biol.* 2002, 9: 95-100


longest
loop
α−1
α0
α6
α1
β6
β0
β3
β5
α5
β1
β2
β4
α3
β8
α4
β7
α3
α2
Sw I
Sw II
α7
N
C
α4
N
C

of leaf development. The mutant *ppi1* plants lacking atToc33 display a delayed greening phenotype but eventually appear similar to wild-type plants, and have reduced levels of chloroplast protein import early in their development (Jarvis et al 1998; Gutensohn et al 2000). Overexpression of Toc33 or Toc34 complements the Toc33 mutant plants, suggesting that the two genes are somewhat redundant.

1.1.2.3 Toc75

Toc75 is the most abundant protein in the outer envelope membrane of the chloroplast and is believed to be the conductance channel for protein translocation (Schnell et al 1994; Tranel et al 1995; Hinnah et al 1997). It traverses the membrane as a series of 16 anti-parallel beta strands which form a beta barrel, and it has cation-selective channel activity (Hinnah et al 1997). It was shown by cross-linking assays that it interacts with preproteins during membrane translocation (Hinnah et al 1997; Schnell et al 1994; Perry & Keegstra 1992), and it stably associates with Toc159, Toc34 and other translocon components, even in the absence of preproteins (Kouranov et al 1998; Nielsen et al 1997; Sohrt & Soll 2000; Schleiff et al 2003). Electrophysiological measurements of Toc75 reconstituted into liposomes indicate a potential channel with a diameter of ~14Å which is large enough to let an unfolded polypeptide chain pass through. It is also able to differentiate chloroplast proteins and other proteins based on the conformational and electrostatic interactions (Hinnah et al 2002).

1.1.2.4 Toc64

Toc64 is the newest component found in the Toc complex (Sohrt & Soll, 2000). It is an integral membrane protein most of whose C-terminus is exposed to the cytosol. The most prominent characteristic of Toc64 is that it has three tetratricopeptide repeat (TPR) domains which mediate protein-protein interaction. This motif has also been found in components of Tom (Translocon in the outer membrane of mitochondria) complexes, Tom70, Tom37, Tom22 and Tom20 (Pfanner et al 1997; Lithgow et al 2000). Tom70, which has seven similar TRP motifs, together with Tom37, is believed to be the first receptor for those preproteins bound by cytosolic factor MSF, a 14-3-3 protein which recognizes and binds phosphorylated presequence of mitochondrial preproteins. The preprotein bound by the guidance complex is thought to be recognized by Toc64 at first, then delivered to the receptor complex composed of Toc86/159, Toc34 and Toc75. The association of Toc64 with the core complex (Schleiff et al 2003) is transient, since it is not present in the core complex isolated from pea chloroplasts.

1.1.2.5 Core complex

As mentioned earlier, there are four major Toc components found in the outer envelope membrane of the pea chloroplast, Toc 159/86, Toc75, Toc34 and Toc64 (Schnell et al 1997; Soll 2002; Schnell & Hebet 2003). Three components, Toc159/86, Toc75 and Toc34, were found to form a stable complex with each other using cross-linking experiments (Schnell et al 1994; Waegemann & Soll 1991), even in the absence of a bound precursor protein (Kouranov et al 1998; Nielsen et al 1997; Seedorf et al 1995; Sohrt et al 2000). Recently, a core Toc complex with an apparent molecular mass ~500kD

was purified from the solublized outer envelope membrane from pea chloroplast (Schleiff et al 2003). Image analysis of transmission electron microscope and biochemical analysis indicates that the core complex obtained was composed of one Toc159, four Toc34 and four or five Toc75 proteins. It forms a finger-like central region which separates four curved translocation channels formed by the four Toc75 proteins in the complex (Figure 1-3). However, the exact location of individual components was not clear in this study. The core complex still showed functional biological activity and the binding of SStp required the presence of GTP (Schleiff et al 2003).

1.1.3 Tic complex

The complete translocation of preprotein into the stroma of the chloroplast requires the assistance of the translocon complex in the inner envelope membrane called Tic (**T**ranslocon in the **i**nner membrane of **c**hloroplast). The Toc and Tic complexes interact cooperatively via contact sites of the membranes so that the preprotein can pass through the two-layer membrane sequentially without any accumulation in the intermembrane space (Nielsen et al 1997; Schnell & Hebert 2003). Although not well characterized, the association of Toc and Tic complexes is believed to be transient.

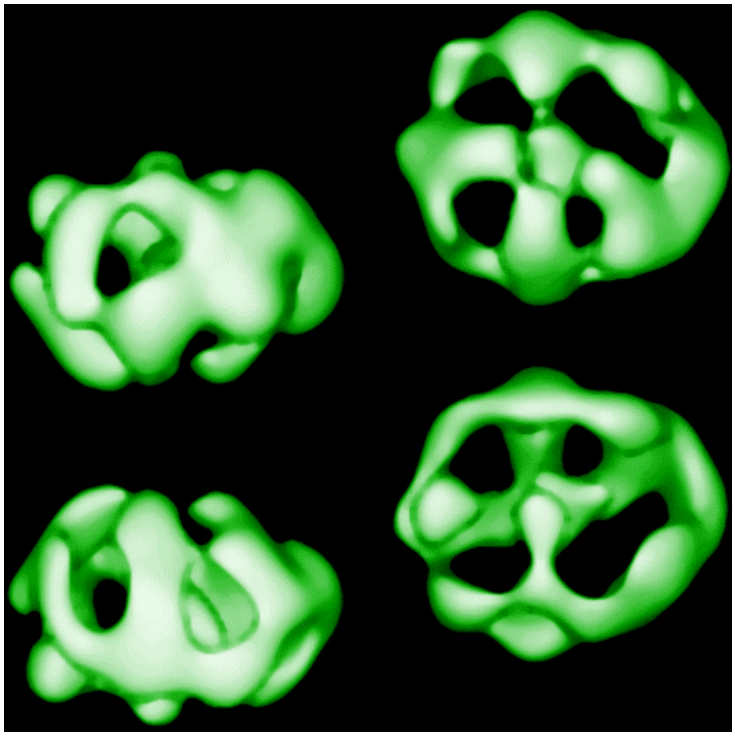
1.1.3.1 Tic110

The first component of the inner membrane import complex to be cloned and characterized was Tic110 (Kessler & Blobel 1996; Lübeck et al 1996). In the inner envelope of the chloroplast membrane, Tic110 is the central unit of the translocon and is

Figure 1-3 Electron microscopy image of the Toc core complex.

The complex was isolated from solubilized outer envelope membrane of pea chloroplast. It has a molecular weight of about 500kD, with a molecular stoichiometry 1:4:4-5 between Toc159, Toc34 and Toc75. The four pores are proposed to be the translocation channels formed by Toc75.

Adapted from Schleiff et al, *J. Cell Biol.* 2003, 160: 541-51.



involved in many events during translocation. It may take part in the formation of contact sites between outer and inner envelope (Nielsen et al 1997), and the assembly of at least a part of the translocation pore (Heins et al 2002). Tic110 has a large domain exposed to the stroma and anchored to the envelope by a membrane-spanning α-helix at the N terminus (Kessler & Bloble 1996; Jackson et al 1998). Based on this topology, it is proposed that it may serve to recruit molecular chaperones like Hsp110 and Hsp60 to the stromal face of the Tic complex (Kessler & Bloble 1996; Nielsen et al 1997; Jackson et al 1998; Bauer et al 2001).

1.1.3.2 Tic40

The observations that Tic40 is associated with precursor proteins arrested during import (Wu et al 1994) and that it is present in cross-linked complexes containing Tic110, suggest that Tic40 is a component of the import apparatus (Stahl et al 1999). Tic40 is resistant to proteases applied exogenously to chloroplasts and isolated envelope membranes (Ko et al 1995) and is predicted to have a single membrane-spanning region at its extreme N terminus (Stahl et al 1999). Tic40 is proposed to recruit molecular chaperone Hsp70 to the site of precursor protein import (Wu et al 1994; Ko et al 1995; Stahl et al 1999), based on its C terminal sequence similarity with Hip (Hsp70-interacting protein). It is essential for plant growth, and the Arabidopsis T-DNA insertion mutants of this single copy gene exhibit defective seedling viability (Budziszewski et al 2001). Ko and coworkers recently found that Tic40 has another related form located in the outer envelope membrane, Toc36. They function independently in the import process, and have

different preferences for different forms of the Oee1 (Oxygen Evolving Enzyme 1) precursor. Tic40 prefers the form containing only the plastid-targeting domain, while Toc36 prefers the form containing both the plastid-targeting and thylakoid lumen-targeting domains (Ko et al 2003).

1.1.3.3 Tic22 and Tic20

It is proposed that Tic110, Tic22, and Tic20 associate with the outer membrane translocon to form the functional contact sites during the translocation process (Chen & Schnell 1999). Tic22 is a hydrophilic protein, localized in the intermembrane space of the chloroplast envelope and appears to be peripherally associated with the inner membrane with no predicted transmembrane domain (Kouranov & Schnell 1997; Kouranov et al 1998). It might function as a receptor when preproteins emerge from Toc and direct the precursors to the inner membrane translocon (Kouranov & Schnell 1997; Kouranov et al 1998). Tic20 is a hydrophobic protein, embedding deeply in the inner envelope membrane and is believed to form at least part of the protein conductance channel in the inner membrane, as suggested by the observation that crosslinking of preproteins to Tic20 increases at the later stages of protein import when the translocating chain has inserted across the inner envelope membrane (Kouranov & Schnell 1997; Kouranov et al 1998).

1.1.4 Involvement of soluble factors

After synthesis of preprotein in the cytosol, the transit peptide is phosphorylated at a specific serine or threonine by a plant-unique protein kinase in the cytosol (Waegemann & Soll 1996). The amino acid sequence found around the phosphorylation site resembles

the phosphopeptide-binding motif for 14-3-3 proteins. This motif starts with a positively charged residue followed by phosphorylated residue (serine or threonine). In the case of prSSU, the serine phosphorylated is the second serine located between the two loosely conserved motifs FGLK (May & Soll 2000). The guidance complex composed of a 14-3-3 dimer and a molecular chaperone Hsp70 recognizes and binds the phosphorylated preprotein and forms a hetero-oligomeric complex which then delivers the preprotein to the preprotein receptors located at the outer envelope membrane of chloroplast (May & Soll 2000; Fulgosi et al 2002). Hsp70 is known to bind nascent chains, keeping them folded or unfolded, aiding in import or targeting competency (Lain et al 1994, Terada et al 1995, Hartl 1996). An outer membrane-associated Hsp70 protein, which faces the intermembrane space of the chloroplast envelope, is believed to interact with precursor protein as they move between the outer and inner membrane translocons (Marshall et al 1990; Schnell et al 1994). The import of prSSU in the presence of the guidance complex shows three to four fold higher translocation speed compared with the free precursor protein in the absence of the guidance complex. It is important to note that *in vitro* experiments have shown that preproteins can still be imported into isolated chloroplasts in the absence of soluble factors at a rate comparable to import under physiological conditions (Cline et al 1993, Dabney-Smith et al 1999, Pilon et al 1992).

1.1.5 Involvement of the lipid component of the envelope membrane

Plastids are semiautonomous organelles with wide structural and functional diversity. They are surrounded by a double-layer membrane which isolates the plastids from the cytosol. The chloroplast membrane has a high lipid/protein ratio (~80%), and therefore

the lipid domain is relatively exposed (Block et al 1983; Douce et al 1984), indicating that the cytosolic surface of the chloroplast is largely lipophilic in nature and the lipid components may play an active role in supporting the function of the chloroplast. The membrane lipids were shown to be involved in the import process by an experiment done by the Soll group, which demonstrated that preprotein binding and import was greatly impaired in those chloroplasts after treatment with phospholipase C (Kerber & Soll 1992).

Plastid membranes contain a large variety of specific lipids, including galactolipids, phospholipids and sulfolipids. PC (phosphotidylcholine) is the building block for the membrane and is the most abundant lipid class in the chloroplast (Joyard et al 1998), Though a lot of experiments have shown that transit peptides interact with liposomes mimicking the outer chloroplast membrane (Kerber & Soll 1992; Pinnaduwage & Bruce 1996; Subramanian et al 1998), interaction of SStp (transit peptide of the precursor to the small subunit of Rubisco) with liposomes composed of PC alone is not observed and the addition of DGDG (digalactosyl diacylglycerol) did not improve binding (Pinnaduwage & Bruce 1996; Subramanian et al 1998). Similar results were obtained for preFd (precursor to ferredoxin) (van't Hof & de Kruijff 1995). Thus it can be concluded that specific lipids other than PC and DGDG are responsible for binding of the precursor to the lipid bilayers.

1.1.5.1 Galactolipids in plants

Two predominant galactolipids are found in higher plant chloroplasts: MGDG (monogalactosyl diacylglycerol) and DGDG (digalactosyl diacylglycerol) (Douce &

Joyard 1990; Gounaris & Barber 1983). A very specific characteristic of the chloroplast outer membrane is that it is the only membrane exposed to the cytosol that contains galactolipids.

MGDG is a polar, cone-shaped molecule with a galactose group at the tip and the two acyl chains extending towards the base of the cone. In aqueous medium, it tends to form a hexagonal-II (H_{II}) phase, with the polar head groups facing inward and the fatty acid chains sticking out (Webb & Green 1991). Because of this morphology, when present in the bilayer, MGDG will increase lateral pressure toward the center of the bilayer with a negative curvature stress (Cantor 1997) and a larger free volume (Mitchell et al 1992). In contrast, DGDG, which has two galactose moieties in its head group, shows a cylindrical shape. It is a bilayer-forming lipid, forming lamellar L_α phases when mixed with water (Dörmann & Benning 2002). In Arabidopsis, three genes responsible for monogalactolipid synthesis are found: *MDG1, MDG2, MDG3.*The MDG1 enzyme has been shown to be the main player in MGDG synthesis in the chloroplast by studies of the insertion mutant of the *MDG1* gene (Jarvis et al 2000).

Since MGDG is a substrate for DGDG synthesis, DGDG synthase (galactolipid: galactolipid galactosyltransferase) is critical in regulating the ratio of non-bilayer lipid to bilayer lipid. It is thought that the ratio is of critical importance for many biological events, like protein folding and insertion (Gounaris & Barber 1983; Bogdanov & Dowhan 1999), protein trafficking (Kusters et al 1994), as well as chloroplast structure and function (Bruce 1998). Studies of an Arabidopsis *dgd1* mutant showed that the import of chloroplast stromal-targeting precursor proteins are impaired (Chen & Li 1998), indicating that DGDG is important for the integrity of the chloroplast protein import

machinery. However, the *dgd1* mutant only showed a reduced amount of DGDG, suggesting that DGDG synthase is not the only enzyme responsible for its synthesis (Dörmann et al 1995).

1.1.5.2 Anionic lipid PG (phosphotidylglycerol)

Another important lipid component in chloroplast envelope membrane is the anionic phospholipid-PG. though it only consists about 10 mol% in the outer membrane (Joyard et al 1991), PG is of great importance for the proper interaction with preproteins (Pilon et al 1995; Horniak et al 1993; Pinnaduwage & Bruce 1996). It is also shown to be essential for the biogenesis and function of thylakoid membranes in analysis of *PGP1* mutants in Arabidopsis (Babiychuk et al 2003). PGP1 and PGP2 are the two PG-phosphotate synthases in Arabidopsis identified and characterized by Müller and Frentzen (2001). PGP1 is a preprotein that can be imported into yeast mitochondria and processed to a catalytically active protein, while PGP2 corresponds to a microsomal enzyme (Müller & Frentzen 2001).

Because of its negatively charged nature, PG is involved in many electrostatic interactions. It is shown to have strong interactions with the basic residues present in the transit peptides as revealed by monolayer experiments (van't Hof & de Kruijff 1995; Pinnaduwage & Bruce 1996). Proteins such as GTPase FtsY associate with membranes in an anionic-dependent manner (de Leeuw & Luirink 1997; de Leeuw et al 2000). Anionic lipids also can influence the polymorphism of the membrane. When cationic peptides insert into the negatively charged lipid, the charges get neutralized and the neutralization results in the compression of the lipid head group and thus increasing the negative

curvature tendency of the membrane (Epand 1998).

1.1.5.3 Structural and chemical features of the bilayer interface

The liquid-crystallographic determination of the structure of fluid DOPC (dioleoylphosphocholine) bilayers suggests that the bilayer can be divided into interfacial and hydrocarbon-core phases (White & Wimley 1998). The interface between the polar aqueous phase and the nonpolar hydrocarbon region of the bilayer is not sharp but rather a transition zone, consisting of a complex mixture of water, glycerol, carbonyl and methylene groups, and it accounts for ~50% of the bilayer thickness (White & Whimley 1998).

Because of the high chemical heterogeneity and high anisotropy of the interfacial layer, the final location of peptides partitioning into the membrane will depend on the balance between hydrophobic, electrostatic, and bilayer effects and will also depend on the conformational states available to the peptide (White & Wimley 1998).

1.1.5.4 Aromatic residues in the interaction with lipids

In the partitioning of peptides into a membrane interface, aromatic residues have a specific role in the process. Studies of the whole residue free energy of transfer ΔG from water to POPC interface showed that aromatic residues have favorable free energies to partition into the membrane interface (White & Wimlley 1998). Trp and Tyr prefer the interface because of their amphipathic nature, the idea being that the aromatic rings reside in the hydrocarbon core with their polar –NH or –OH groups forming hydrogen bonds with lipid carbonyls (Schiffer et al 1992). In addition, the steric bulk of Trp when located

deeper in the membrane can promote the formation of inverted phase (Killian et al 1996), which would change the organization of the membrane.

1.1.6 Signal processing peptidase

The last step of protein import into the chloroplast is the removal of the transit peptide from the mature protein. It has been shown that the transit peptide is cleaved off by a stromal processing peptidase (SPP), which is a metalloendopeptidase, during or after translocation (de Boer & Weisbeek 1991; Oblong & Lamppa 1992; VanderVere et al 1995). Analysis of the amino acid specificity for SPP in the vicinity of the processing site of 32 precursors that are targeted to the stroma indicated a loosely conserved motif consisting of (Val/Ile)-X-(Ala/Cys) $^{\downarrow}$A, X can be any amino acid (Gavel & von Heijne 1990). After the removal of the transit peptide, the mature protein begins the process of folding and assembly (Oblong & Lamppa 1992; VanderVere et al 1995; Richter & Lamppa 1998).

1.2 Rubisco

Rubisco stands for ribulose 1,5 bisphosphate(RuBP) carboxylase/oxygenase. It is the most abundant protein in the world, representing about 50% of soluble chloroplast protein and 15-20% of soluble protein in green cells (Spreitzer & Salvucci 2002). Rubisco catalyzes the first step in net photosynthetic CO_2 assimilation and photorespiratory carbon oxidation. The functional form of Rubisco is composed of 8 large subunits encoded by the plastid genome and 8 small subunits encoded by the nuclear genome (Dean et al 1989; Spreitzer 1993). After synthesis on the free ribosomes

in the cytosol, prSSU (**pr**ecursor to the **s**mall **s**ubunit of **Ru**bisco) is transported back into the chloroplast stroma (Figure 1-1), and there it forms the holoenzyme with the large subunit (Spreitzer 1999). prSSU is the first chloroplast protein that has been shown to be targeted back into chloroplast and is the most extensively studied chloroplast precursor in the literature.

1.3 Transit peptide

Proteins synthesized in the cytoplasm have different destinations, some remain in the cytoplasm or in a topologically similar place like the endoplasmic reticulum (ER); others are transported to other organelles like mitochondria, chloroplasts etc. For transport to organelles, there is always a peptide located in the N-terminal end of the precursor protein that is responsible for targeting the precursor to those organelles. These transit peptides will eventually be cleaved off and are not present in the mature protein.

1.3.1 Features of transit peptides

Transit peptides of different chloroplast proteins vary greatly (Keegstra et al 1989), they are highly heterogeneous in length, composition and organization (Bruce 2001). Only the sequence of the first two amino acids, MA, is well conserved among the transit peptides (Pilon et al 1995). The length of transit peptide ranges from 13 to 146 amino acids and the average length is about 58 amino acids (Zhang & Glaser 2002). In general, transit peptides are rich in hydroxylated amino acids (serine and threonine) and small hydrophobic residues. Although they are not particularly rich in basic residues (arginine

and lysine), they tend to lack acidic amino acids (aspartate and glutamate) (Keegstra et al 1989).

1.3.2 Structure of the transit peptide

Thousands of different proteins are predicted to be targeted to plastids via a transit peptide (Initiative 2000), yet only a limited number have been experimentally studied (Perry et al 1991; Row & Gray 2001). These proteins include the precursor of the light-harvesting chlorophyll a/b-binding protein (pHLCP), the precursor of ferredoxin (prFd), the precursor of plastocyanin (pPC), the precursor of the small subunit of Rubisco (prSSU) (Perry et al 1991), the precursor of the 33 kDa subunit of the oxygen-evolving complex (pOE33), ferredoxin $NADP^+$ reductase (pFNR), porphorbilinogen deaminase, the Rieske iron-sulphur protein (pRieske protein), ferrochelatase, the γ subunit of the ATP synthase and phosphate translocator (Row & Gray 2001). These results suggest that all of these precursor proteins compete for a common pathway for import. However, the transit peptides that are responsible for guiding these preproteins into chloroplast do not share much amino acid sequence homology.

It has been suggested that interaction between the transit peptide and chloroplast envelope membrane lipids give rise to recognizable structural motifs. Previous studies show that transit peptide alone from both prSSU and preFd can interact directly with the outer membrane lipids (Pilon et al 1995; Dabney-Smith et al 1999; Subramanian et al 2001) and interact functionally with the general import machinery (Perry et al 1991; van't Hof et al 1995; van't Hof et al 1993; Pilon et al 1995; Dabney-Smith et al 1999; Subramanian et al 2001). These observations lend credibility to the use of the isolated

transit peptide to study the structural behavior upon its association with membranes.

Based on early NMR work (Horniak et al 1993; Wienk et al 1999), the structure of Silene trFd (transit peptide of the precursor to ferredoxin) in different environments was summarized by Bruce(Bruce 2001). It is totally unstructured and fully extended in an aqueous solution. In TFE (trifluroethanol)/H_2O, large helices are introduced at both N- and C- terminal ends, and a small helix appears in the middle. In the presence of MGDG/DOPC/DOPG micelles, helices are promoted in the same regions, but to a lesser extent. The FGLK motif is found at the end of the small helix in the middle region. NMR study on synthetic peptide SStp (transit peptide for prSSU) revealed the same structure: both N- and C-termini exhibit α-helices with a central region largely unstructured (Bruce 1998).

1.3.3 FGLK motif

Transit peptides show high heterogeneity among different precursor proteins (Keegstra et al 1989), and only a loosely conserved FGLK motif has been identified in many transit peptides of different chloroplast preproteins (Pilon et al 1995). However, when the same preprotein from different organisms were compared, significant regions of higher homology can be identified. For instance, one FGLK motif can be found in all the prFds from five different organisms (Pilon et al 1995) and two similar motifs are conserved in all the fifteen different prSSU sequences as shown in chapter 3. Using bioinformatics, we are going to look at more proteins to characterize this motif in chapter 3.

Earlier work has shown that this FGLK motif contributed to preprotein recognition in the protein import into chloroplasts. Analysis of Silene trFd deletion mutant indicated that

FGLK domain might contribute to targeting (Pilon et al 1995). It is also of interest to note that the synthetic peptides that were shown to inhibit chloroplast import and binding of a set of *in vitro* translated precursor proteins, all shared a similar motif FGLK (Schnell et al 1991; Perry et al 1991). NMR studies revealed that the loosely-conserved FGLK motif shows a trend to form secondary structure in aqueous medium, and forms the most stable helical structure in the presence of either 50% TFE or micelles (Wienk et al 1999; Wienk et al 2000). All this evidence indicate that the structural behavior of this region may assist in correct positioning of a recognition motif in the chloroplast membrane, or itself is a critical recognition element for the translocation machinery (Wienk et al 2000; Bruce 2001).

1.4 Summary

Protein import into chloroplasts is a critical process for the proper function of this photosynthetic organelle. Work in this lab mainly focuses on the study of the early event in import: the interaction of preprotein with membrane lipids and preprotein receptors at the outer envelope membrane of the chloroplast. Interested in identifying the roles of the semi-conserved FGLK motifs identified in the transit peptide of tobacco prSSU, F_{26}TGLK and F_{35}PVSRK, we have mutated the aromatic components of the two motifs to study their function in the protein import process, especially in the preprotein interaction with membrane lipids. Characterization of the semi-conserved FGLK motifs in the transit peptide of prSSU will provide some insights into understanding this import process.

Chapter 2 Materials and methods

2.1 Plant growth and chloroplast isolation

Dwarf pea (*Pisum sativum*) was used as a model plant and chloroplasts were isolated from pea seedlings that were 14 days old. Approximately 300 ml of dry pea seeds (Laxton Progress #9) were soaked overnight with aeration and planted on horticultural Vermiculite (Palmetto Coarse A3, Woodruff, SC) on each flat (12 × 24 inch). Plants were grown in EGC growth chambers under 160 μE/m^2/sec of cool white fluorescent light. The plants were grown on a 12 hr light/12 hr dark cycle to reduce starch accumulation. Leaves were harvested at the beginning of the light cycle and pulse-chopped in a Cuisinart food processor for very short bursts (2-3 sec each) until they were homogeneously mixed. The chopped tissue was then moved to an electric polytron and homogenized in the presence of ~500 ml of Grinding Buffer (GB: 330 mM Sorbitol, 2 mM $MgCl_2$, 2 mM MnCl2, 4 mM EDTA, 0.2% BSA, 50 mM HEPES-KOH, pH 7.3) in 2-3 sec bursts 3-4 times. The amount (ml) of GB used for grinding is 3 times the mass (mg) of the leaves harvested. The resulting homogenate was filtered through 4 layers of cheesecloth and 1 layer of MiraCloth. The filtrate was centrifuged at 2,000 × g for 6 min at 4℃ and the supernatant was discarded. The pellet was resuspended in 15-20 ml of 1 × Import Buffer (IB: 33 mM Sorbitol, 50 mM HEPES-KOH, pH 8.0) and was then gently layed onto a 50% Percoll (Pharmacia) continuous gradient. Percoll gradients were prepared by mixing equal volumes of 2 × IB and 100% Percoll and spun for 30 min at 20,000 × g with the centrifuge brake off. The loaded gradients were centrifuged at 5,800

× g for 14 min with the brake off. The lowest dark-green band of intact chloroplasts was collected using a large-bore syringe needle. The intact chloroplast fractions were combined with about 3 × volume 1 × IB by mixing first and then spinning down at 3,500 × g for 6 min to remove Percoll. Intact chloroplasts were resuspended with ~ 2 ml 1 × IB.

2.2 Chlorophyll measurement

Chlorophyll concentration was measured after chlorophyll extraction using 80% acetone. To calculate the total mg of chlorophyll in the chloroplast preparation, 50 µl of the chloroplast suspension was added to 5 ml of 80% acetone and spun at 14, 000 × g for 3 min to remove the starch granules. Absorbance at 663 nm and 645 nm were read and used in the following equation to calculate chlorophyll content (Bruce et al 1994).

$$Chlorophyll\ (mg/ml) = \frac{\left[8.65 \times A_{663} + 20.2 \times A_{645}\right]}{0.05 \times 1000} \times 5ml$$

Chloroplast resuspension was further diluted with 1 × IB to get the concentration of 1 mg chlorophyll/ml and used for import reactions.

2.3 Protein measurement

Protein concentration was determined using BCA (bicinchoninic acid) reagents (Pierce, Rockford, IL). Bovine serum albumin (BSA, Pierce, Rockford, IL) was used as the standard protein. For protein solutions that have reducing reagent DTT or β-ME, proteins were first treated with Compat-Able kit (Pierce, Rockford, IL) to precipitate protein and

then regular BCA protein assay was performed.

2.4 Preparation of competent cells

E. coli strains XL-Blue and BL21 [DE3] RIL were used for plasmid prep and protein expression, respectively. Competent cells were made for transformation.

An LB agar plate without antibiotic is streaked with cell strain glycerol stock and grown overnight. A preculture is grown overnight and 1% volume of this overnight culture is used to inoculate an LB medium prewarmed to 37℃. Cells are put on ice for 15 min when OD_{600} reaches 0.4. Then cells are transferred to an autoclaved 250 ml centrifuge bottle and are centrifuged at 10,000 × g for 5 min. The cell pellet is resuspended very gently in 50 ml wash buffer (5 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.6) and is centrifuged again. The cell pellet is washed with wash buffer again and resuspended in 25 ml Calcium Buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM $CaCl_2$, pH 7.6). The cells are spun down after they are stored on ice for 30 min. Then the cell pellet is resuspended gently with 4 ml Storage Buffer (10 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 100 mM $CaCl_2$, 7% (v/v) glycerol). 100 µl aliquots were stored in sterile microcentrifuge tubes at -80℃.

2.5 Generation of F to W/A/S mutants

Multi-quik site-directed mutagenesis of prSSU was performed using Stratagene QuikChange multi site-directed mutagenesis kit (Stratagene, La Jolla, CA). Single-strand primers were designed according to the mutation sites and were synthesized at IDT

(Coralville, IA). Sequences of the primers are given in Table 2-1. Single site-directed mutagenesis was also used. Primers are shown in Table 2-2. Dpn I (Madison, WI) was used to digest template DNA at 37℃ for an hour after PCR reaction. Annealing temperature (Tm) for the primers was calculated following the formula below:

Tm= 81.5 +0.41 (% GC) -675/N -% Mismatch

- N is the length of the primer in bases
- Values for % GC and % mismatch are whole numbers

PCR reaction was performed in an Eppendorf thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) with 6 primers (total 200 ng) mixed in each PCR reaction tube, 3 for each site, and in equimolar amounts. The PCR reaction was carried out as below:

Step 1 Denaturation: 94℃ 4 min;

Step 2 Denaturation: 94℃ 1 min;
Step 3 Annealing: 55℃ 1 min;
Step 4 Extension: 65℃ 14 min;
} 30 cycles

Step 5 Extension: 65℃ 7 min;

Step 6 Storage: 4℃

The enzyme used in this multi-quik site directed mutagenesis is a mixture of polymerase and ligase. The ligase links together the fragments generated in the PCR cycle and then another cycle starts. The PCR product was digested with Dpn I enzyme to digest methylated and hemimethylated DNA at 37℃ for an hour. Then the digested product was transformed into Golden XL-10 competent cells (~10^8 colonies/μg DNA) following

Table 2-1 Primer sequences for multi site-directed mutagenesis

primer name	length (bases)	sequence 5'→ 3'
F26W	27	ATG GTT GCA CCT **TGG** ACT GGC CTT AAG
F26A	27	ATG GTT GCA CCT **TCC** ACT GGC CTT AAG
F26S	27	ATG GTT GCA CCT **GCC** ACT GGC CTT AAG
F35W	27	TCA GCT GCC TCA **TGG** CCT GTT TCA AGG
F35A	27	TCA GCT GCC TCA **TCC** CCT GTT TCA AGG
F35S	27	TCA GCT GCC TCA **GCC** CCT GTT TCA AGG

Table 2-2 Primer sequences for single site-directed mutagenesis

primer name	strand	length	sequence 5'→3'
F35W	+	27	TCA GCT GCC TCA **TGG** CCT GTT TCA AGG
F35W	-	27	CCT TGA AAC AGG **CCA** TGA GGC AGC TGA
F35S	+	27	TCA GCT GCC TCA **AGG** CCT GTT TCA AGG
F35S	-	27	CCT TGA AAC AGG **GGT** TGA GGC AGC TGA

the protocol provided by the kit. Figure 2-1 shows the overview of the QuikChange Multi site-directed Mutagenesis method.

~ 100 colonies were picked and grown in 10 ml LB/Amp 150 μg/ml overnight. DNA was purified using Wizard vacuum mini-prep kit from Promega (Madison, WI). The presence of mutation was confirmed by automated sequencing (Macrogen, Korea). For single site-directed mutagenesis, the same PCR reaction condition was used except that only 2 regular primers (100 pm) were in each reaction tube and the Taq polymerase pfu used was from Promega (Madison, WI). PCR product was transformed to XL-Blue competent cells after Dpn I treatment. Plasmids were extracted using Wizard vacuum mini-prep kit from Promega (Madison, WI) and confirmed by sequencing (Macrogen, Korea).

2.6 Purification of prSSU Phe mutants from inclusion bodies

Plasmids containing mutated prSSU were transformed into BL21 [DE3] RIL following normal transformation protocol. Several colonies were inoculated into 10 ml LB (Amp 150 μg/ml) medium respectively, and were grown at 37℃ until OD_{600} reached 0.6. Then 1 ml culture was transferred to a new tube and 1 μl 1 M IPTG (isopropyl-β-D -thiogalactoside) was added for induction. An 18% polyacrylamide gel was run to determine which colony had the best expression and the remaining uninduced preculture (9 mls) was used as inoculum for 1 L LB/Amp 150 μg/ml medium. IPTG was added when OD_{600} reached ~0.6 and the cells were grown for 3 more hours. The cells were then spun down at 5,000 × g for 10 min and the supernatant was decanted. The

Figure 2-1 Overview of the QuikChange Multi Site-Directed mutagenesis method.

A. Step 1: Mutant strand synthesis (Thermal cycling). Perform thermal cycling to:

1) Denature template DNA

2) Anneal mutagenic primers (all primers bind to the same strand)

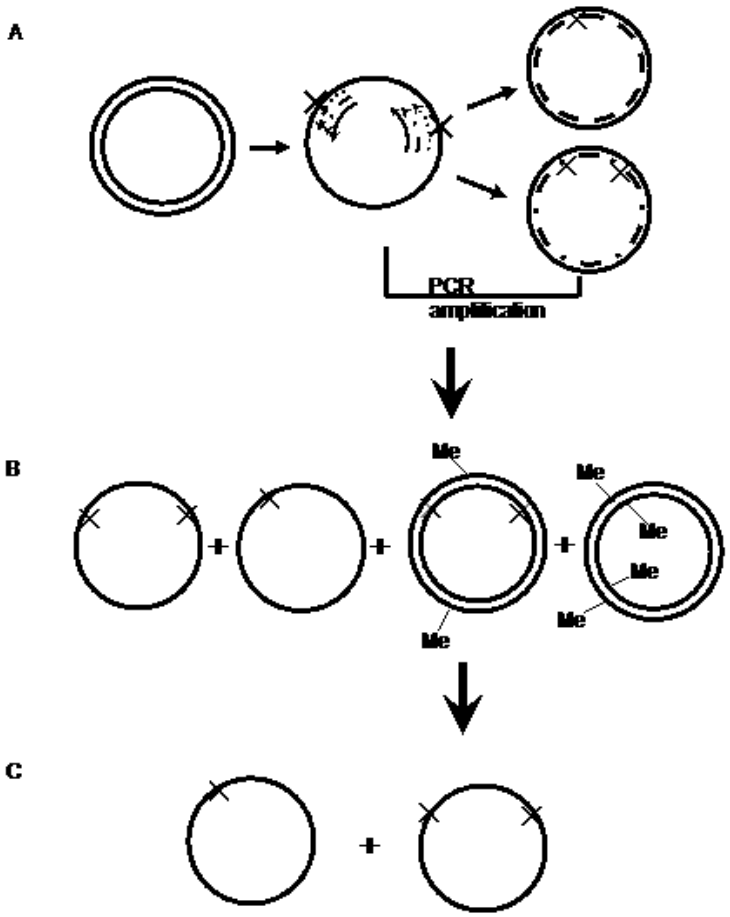
3) Extend primers and ligate nicks with the QuikChange Multi enzyme blend

B. Step 2: Dpn I digestion of methylated and hemimethylated DNA

C. Step 3: Transformation. Transform mutated ssDNA into XL10-Gold ultracompetent cells.

Different format of lines with arrow end depict primers containing different substitution amino acid for a given mutation site.

"×" stands for the mutation site.


A
PCR
amplification
B
Me
Me
Me
Me
Me
Me
C

pellet was resuspended in 30 ml 1× Buffer A (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.6) and spun down at 40,000 × g for 10 min. Pellets were frozen at -80 ℃. Then the next day pellets were resuspended in 30 ml 1 × Buffer A+ 0.1% Triton X-100 and sonicated (Misonix Sonic 550 probe ultrasonicator) for 4 min with 10 sec on and 10 sec off. The samples were then centrifuged at 40,000 × g for 10 min. The supernatant was decanted and 20 µl was saved for gel analysis. Then the pellet was resuspended with 30 ml 1 × Buffer A+ 0.1% Triton X-100 and treated with sonication and centrifugation as before. This step was repeated at least 2 more times. The pellet was then rinsed with 30 ml 1× Buffer A 3 times to remove Triton X-100. The final inclusion body pellet was incubated with 8 M urea + 50 mM DTT at room temperature overnight and then was centrifuged at 40,000 × g for 30 min. Supernatant was collected and protein concentration was determined by BCA assay. 200 µl aliquots at 2 mg/ml were made and frozen at -80℃.

2.7 In vivo *radiolabeling of wild type and mutant prSSU*

E. coli BL21 [DE3] RIL strain was used for generating ^{35}S-radiolabeled wild type (WT) and mutant prSSU proteins. A screening gel was first run to select the colony that had the best expression of the desired protein. The selected colony was first grown in regular LB medium, when OD_{600} reached ~0.6, the cells were spun down and washed twice with Dulbecco's Modified EAGLES Medium (DMEM, BioWhittaker) without cysteine or methionine. Then the cells were grown in DMEM medium for about 4 hours before 1 mM IPTG (final concentration) was added. Cells were induced for 15 min, then Tran^{35}S-Label metabolic labeling reagent (ICN) was put in. The cells were induced for

another 3 hours before they were spun down. The inclusion bodies were isolated using the same method described in section 2.6 and solubilized in 8 M urea + 50 mM DTT. Specific activity was calculated which was always 2×10^6 dpm/μg protein or greater, and it was corrected for the purity of the protein.

2.8 In vitro *protein import competition assay*

Import competition assay was performed with freshly made chloroplast (25 μg/ml chlorophyll) by incubating 100 nM ^{35}S-prSSU and increasing concentration of cold competitors (0 nM, 100 nM, 300 nM, and 600 nM) in the presence of 3 mM Mg-ATP and 10 mM DTT (see Table 2-3). The urea concentration was kept below 500 mM, so that it will not affect import (Cline et al 1993). To start the assay, ^{35}S-prSSU and competitor were added simultaneously and the reaction was incubated at room temperature for 20 min with occasional inversion. The assay was terminated by adding 700 μl ice-cold 1× IB and the tube was immediately put on ice. The sample was then layered over 40% percoll (for 100ml: 50 ml 2 × IB, 40 ml 100% percoll, and 10 ml H_2O). Intact chloroplasts were reisolated using a swinging bucket at low speed (3,000 × g) and resuspended with 1 ml 1 × IB. 50 μl was taken out for BCA assay and the other 950 μl was spun down as in the previous step. Pellet was resuspended again with 25 μl ddH_2O and vortexed vigorously. Sample was boiled for 3 min with 25 μl 4 × SSB (sample solving buffer) and frozen at -20□ for slab polyacrylamide gel. Equal amount of proteins were loaded on each lane for the gel. After the dye front was run off the bottom edge, the gel was stopped running and was dried on a gel-dryer (BioRad, Richmond, CA). Then filmless autoradiography

Table 2-3 Chloroplast protein import competition assay setup.

300 μl reaction / μl	Competitor concentration (nM)			
	0	100	300	600
1 × IB	195	195	195	195
2 × IB	14.8	14.8	14.8	14.8
DTT (1 M)	3	3	3	3
Mg-ATP(100 mM)	9	9	9	9
Chloroplast (1 mg chlorophyll/ml)	75	75	75	75
35S-WT-prSSU (6.25 μM)	4.8	4.8	4.8	4.8
Competitor (32 μM)	0	0.94	2.8	5.0

was performed (Instant-*Imager,* Packard Instruments) and quantification of signal was analyzed by Instant*Imager* analysis software (Packard Instruments).

2.9 Data analysis

Data analysis was performed by electronic, filmless autoradiography (Instant-*Imager*, Packard Instruments), and quantification of signal by Instant *Imager* analysis software (Packard Instruments). Signals were reported as cpm's and converted to molecules mSSU/chloroplast/min using the counting efficiency of the instrument, the specific activity of the precursor, the cysteines and methionines per molecule, the number of chloroplasts per ml import reaction, and the length of import time. The counting efficiency of the Instant*Imager* was calculated relative to measurements made using a liquid scintillation counter using the formula

$$Efficiency = \frac{net\ CPM\ \ prSSU\ \ I.I.}{net\ DPM\ \ prSSU\ \ L.S.C.}$$ (0.01 in the calculation)

using a known amount of protein. The counting efficiency of the Instant*Imager* was established to be 0.01, and was used to determine DPM of a sample which could be converted to DPM/μg precursor/min. The data was analyzed using GraphPad Prism™ (GraphPad Software, Inc.) computer software using non-linear regression. The following calculations were performed unless otherwise noted.

Converting CPMs (from Instant Imager) to DPMs

$$DPM = \frac{CPM\ \ prSSU}{Efficiency}$$

whenever a new prSSU prep is made， Specific Activity need to be calculated:

$$SA\ (DPM/\mu g) = \left[\frac{DPM/\mu l}{\mu g/\mu l\ protein}\right]$$

$$DPM/\mu l = \left(\frac{total\ DPM}{\mu L}\right) * \%\ prSSU/lane$$

To calculate molecules mSSU/DPM

$$\left[\left(\frac{DPM}{\mu g}\right)\left(\frac{20300\ g}{mole}\right)\left(\frac{1\times10^{6}\ \mu g}{1\ g}\right)\left(\frac{1\ mole}{6.022\times10^{23}\ molecules}\right)\right]^{-1} = \left(\frac{DPM}{molecule}\right)^{-1}$$

$$\left(\frac{molecules\ mSSU}{DPM}\right) = \left(\frac{molecules}{DPM}\right)\frac{9 sites/prSSU}{5 sites/mSSU}$$

To calculate DPM/µg protein loaded on gel/min, need to know µg protein loaded onto gel from BCA assay and reaction time of assay (in min.)

$$\frac{DPM/\mu g}{\min} = \left(\frac{DPM\ in\ lane}{\mu g\ loaded}\right)\left(\frac{1}{time\ (\min)}\right)$$

To calculate Chloroplasts(cts)/ml

Volume of grid square on hemocytometer = $0.2\ mm \times 0.2\ mm \times 0.1\ mm$

$$= \left(0.004\ mm^{3}\right) * \left(\frac{1\ ml}{1000\ mm^{3}}\right)$$

$$= 0.000004\ ml$$

$$\frac{cts}{ml} = \left(\frac{Avg\ count/grid}{0.000004\ ml}\right) * d.f.$$ d.f.=dilution factor

To calculate mg cts protein/mL (to convert cts/mL to cts/µg protein and ultimately to calculate cts/lane on gel) do a BCA of chloroplasts diluted say 1:10 and 1:5 and average.

To calculate number of chloroplasts per µg protein:

$$\frac{chloroplasts}{\mu g\ protein} = \left(\frac{cts}{mL}\right)\left(\frac{1}{mg\ cts\ prot/mL}\right)\left(\frac{1mg}{1000\mu g}\right)$$

To calculate the number of chloroplasts loaded per lane on gel:

$$\frac{cts}{lane} = \left(\frac{cts}{\mu g\ prot}\right) * \mu g\ loaded$$

To calculate number of molecules mSSU per assay per min:

$$\frac{molecules\ mSSU \big/ assay}{\min} = \left[DPM * \left(\frac{molecules\ mSSU}{DPM}\right)\right] \div time$$

To calculate molecules mSSU per chloroplast per minute:

$$\frac{molecules\ mSSU \big/ cts}{\min} = \left(\frac{molecules\ mSSU \big/ assay(lane)}{\min}\right) \div \left(\frac{cts}{lane}\right)$$

2.10 Subcloning of mutant SStp into pTYB2 (IMPACT-CN) vector

pET11d-prSSU plasmids containing the mutation site(s) were used as the templates for the PCR amplification. Primers corresponding to regions flanking the SStp-encoding region were used to amplify the insert via standard PCR protocol that engineered Nde I and Xma I restriction sites at the 5' and 3' ends of the amplified product. The sense primer used was 5'- GGT AGA TA<u>C ATA TGG</u> CTT CCT CAG TTC-3' and the anti-sense primer used was 5'- GTT AAT TGG TGG <u>CCC GGG</u> CTG CAT GC-3'. The SStp genes amplified from the pET11d-prSSU constructs were double digested with Nde I and Xma I restriction enzymes (NewEngland Biolabs, Beverly, MA) at 37℃ for 3 hours. The digested product and $pTYB_2$ plasmid (NewEngland Biolabs, Beverly, MA)

Nde I

Xma I

were gel purified using QIAquick PCR gel purification kit (Qiagen, Valencia, CA). Ligation was performed using approximate 10:1 insert: vector molar ratio and the ligase used was from Promega (Madison, WI). The ligation reaction was done at room temperature overnight. Ligation product was transformed into competent XL-Blue *E. coli* cells following normal transformation protocol.

2.11 Direct colony PCR screening of ligation product

The presence of SStp gene in $pTYB_2$ vector was first confirmed by colony screening. 25 µl reactions were set up containing Takara ExTaq polymerase (Madison, WI) and the PCR reaction was performed in an Eppendorf thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany). The colony PCR primers used were T7 universal primer: 5'-T AAT ACG ACT CAC TAT A -3' and intein reverse primer: 5'-GAG GTT GGT AAT AAG GTC ATG GGT-3'. At the same time, a patch plate was created. 6% polyacrylamide gel was run for the PCR products and positive colonies were grown up from the patch plate according to the fragment size on the gel (349 bp was desirable). Plasmids were purified and sequencing confirmed as mentioned in section 2.5.

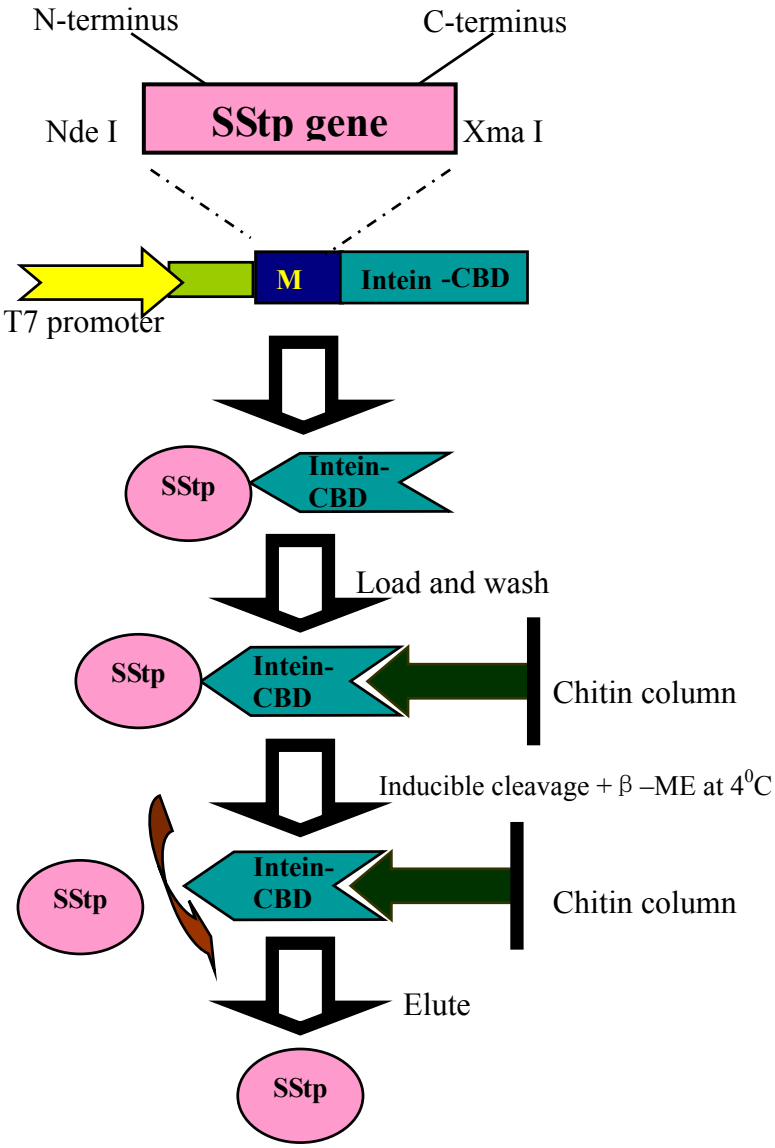
2.12 Expression and purification of SStp mutants from E. coli cells

The construct pTYB2-SStp placed a splicing element that has an inducible self-cleavage activity (termed intein, from *Saccharomyces cerevisiae* VMA1 gene) and a chitin binding domain (from *Bacillus circulans*) at the C-terminus of SStp. The novel purification system allows the purification of full length SStp without the involvement of an external protease and the purification procedure is outlined in Figure 2-2. The plasmids containing

Figure 2-2 Schematic illustration of the IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag)-CN system

The target gene-*SStp* was put into the pTYB2 vector through Nde I and Xma I sites at the multicloning sites and intein tag was fused to the C-terminus. After expression in the host cell ER2566, cells were lysed and supernatant was loaded onto the chitin column. The expressed protein SStp binds to the chitin column through the intein tag. When reducing reagent like β-ME is present, it induces the self-cleavage activity of intein, SStp was eluted off while intein still remains bound to the chitin column.

M is the multicloning site of the pTYB2 vector.


N-terminus
C-terminus
Nde I
SStp gene
Xma I
M
Intein -CBD
T7 promoter
SStp
Intein-
CBD
Load and wash
SStp
Intein-
CBD
Chitin column
Inducible cleavage + β –ME at 4^{0}C
SStp
Intein-
CBD
Chitin column
Elute
SStp

the mutant SStp gene was transformed into *E. coli* ER 2566 cell line. The cells were grown at 30℃ to an OD_{600} of 0.6, induced with 1 mM IPTG (isopropylthio-β-D galactoside) for 3 hours. Cells were spun down using GS-3 rotor at 5,000 rpm for 10 min and pellets were frozen at -20℃.

Cell pellets were resuspended in 30 ml Lysis Buffer (20 mM Hepes pH 8.0, 300 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) and French pressed once. Then the cell solution was sonicated the same way as the full length mutant described earlier in section 2.6. After sonication, the solution was spun down at 20, 000 × g for 10 min. The supernatant was saved and the pellet was resuspended with lysis buffer and sonicated again. After centrifugation, the 2nd supernatant was pooled together with the first one and treated with Benzonase (Novagen, Madison, WI) on ice for 15 min. For 1liter culture, 2 µl Benzonase were used. After centrifugation, the supernatant was loaded into the chitin column that was prepacked and washed thoroughly with at least 5 column volumes of column buffer (20 mM Hepes pH 8.0, 300 mM NaCl, 1 mM EDTA). Flow through was collected for gel analysis and column was washed with 10 column volumes of column buffer. To begin on-column cleavage, the column was quickly flushed with 3 bed volumes of the elution buffer (column buffer + 50 mM β-ME). This process should last no longer than 30 min. Then the column was capped off and incubated at 4℃ overnight. Proteins were eluted off and collected. Gel and protein assays were performed to investigate the purity and concentration. Proteins were lyophilized and stored in powder form at -80℃.

2.13 SDS-PAGE and Coomassie Brilliant Blue staining

Proteins were first separated by SDS-PAGE (5% acrylamide-stacking and 18% acrylamide-separating) and stained in Coomassie staining solution (10% acetic acid, 50% methanol, 0.25% Coomassie Brilliant Blue R 250) for half an hour and destained in destaining buffer (10% acetic acid, 50% methanol) for 30 min. Finally pictures were taken using UVP BioImaging Systems (Upland, CA).

A 19.2% Tris/tricine gel was used for SStp separation because of its better resolution. The recipe for buffers and gel solution were listed in Table 2-4. For a mini gel, 5 ml of resolving gel and 3 ml of spacer gel were mixed separately, 25 μl 10% APS and 10 μl TEMED were added to each of the gel solution. The gel solutions were mixed and poured quickly. Resolving gel was poured first to about 4 cm high, then 1 cm spacer was carefully layered on top of the resolving gel. The glycerol in the resolving gel should keep them from mixing. After the resolving gel and spacer have polymerized, the stacking gel was poured (4 ml stacking solution were mixed with 40 μl 10% APS and 10 μl TEMED). 1 × cathode (upper) and anode (lower) buffers were used to run the gel.

2.14 Liposome preparation

Lipid vesicles were prepared by mixing appropriate amounts of individual lipids (10 mM total) dissolved in chloroform:methanol mixture (2:1). OM liposome was composed of MGDG, DGDG, PC and PG with a molar ratio of 20: 30: 40: 10. PC/PG liposomes were made of PC and PG with a molar ration 60/40. PC liposome was made of 100% PC. The solvent was evaporated at 45℃ under a stream of nitrogen gas for 30 min and the

Table 2-4 Recipe for 19.2% Tris/Tricine gel

10 × Anode Buffer/L	10 × Cathode Buffer/L	Gel buffer/L
242 g Tris	121 g Tris	181.5 g Tris
pH to 8.9 w/HCl	pH to 8.5 w/HCl	pH to 8.45 w/HCl
10 g SDS	179 g Tricine	1.5 g SDS

Gel solutions (Store cold and use within a few weeks). Make these in 50 ml Falcon tubes.

Solution	40% Acrylamide (29:1)	Gel Buffer	80% Glycerol
5% Stack	5	10	-
10% Spacer	10	13.3	-
19.2% Resolving	19.2	13.3	7

Use H_2O to add up to 40 ml each.

samples were vacuum desiccated overnight to remove any trace solvents. The dried lipid film was rehydrated in 0.1 × phosphate-buffered saline (PBS: 1.7 μM K_2HPO_4, 50 μM KH_2PO_4, 15 μM NaCl, pH 7.4) containing 1 mM EGTA, 0.02% azide and 200 mM calcein (final concentration 50 mM). The mixture was vortexed vigorously to remove film of lipids from the surface of the tube. Small unilamellar vesicles were made by sonication using a bath sonicator (Laboratory Supplies Inc.) for 5 min. At least two additional sonication events were performed with an hour interval until the lipid mixture was clear, indicative of formation of small liposomes. Free unincorporated calcein was removed by size exclusion chromotagraphy on a Bio-Gel A0.05m mesh 200-400 column equilibrated with the 0.1 × PBS/1 mM EGTA/0.02% azide buffer. Liposomes were eluted in the void volume fractions and different fractions were collected.

For liposomes without calcein enclosed, the procedure is the same as the steps described above, except that lipid film is rehydrated with 0.1 × PBS alone and no column is needed.

2.15 Fluorescence quenching measurement

The intactness of liposome with calcein entrapped was determined by the fluorescence quenching measurement that was carried out using the Perkin Elmer LS 50B Luminescence Spectrometer (Foster City, CA). The calculation was done using the formula below:

$$\%\ Quench = \left(1 - \frac{F_o}{F_t}\right) \times 100\%$$

F_o and F_t are the fluorescence of the liposome sample before and after addition of 0.1% Triton X-100, respectively. Only fractions showing a calcein fluorescence quenching of >70-80% were used in the calcein dye release assay.

2.16 Protein-induced release of calcein from liposomes

Fluorescence of liposomes was measured in a solid 96-well glass plate (Konte glass, Germany) using a Wallac Victor^2 1420 Multi label counter (Turku, Finland) fluorescence plate reader. Excitation wavelength was set at 485 nm using a fluorescein excitation filter and emission was set at 514.5 nm with narrow band pass filter (10 nm). Liposomes containing entrapped calcein were incubated at room temperature for 1 hour with various concentrations of different proteins as indicated in the respective figure legends. The fluorescence measurements were performed as described above. The percentage of calcein release was calculated using the formula below.

$$\%\ \mathrm{Re}\,lease = \frac{F - F_o}{F_t - F_o} \times 100\%$$

F_o and F are the fluorescence before and after addition of the protein, respectively, and F_t is the total fluorescence after addition of 0.1% Triton X-100 (final concentration). All measurements were repeated at least four times and data was analyzed using GraphPad Prism software.

2.17 Tryptophan fluorescence spectroscopy

Tryptophan fluorescence was measured on a Perkin Elmer LS 50B Luminescence Spectrometer (Foster City, CA) with FL Data Management software. All measurements were taken in 1×1 cm quartz cuvettes at room temperature. 2 ml protein solution (1 μM in 50 mM Hepes pH 7.6, 0.5 mM DTT and 1‰ TFA) was titrated in with different amount of liposomes manually. The fluorescence was excited at 280 nm and the emission scanned from 290 to 420 nm. Scans were taken with 5 nm excitation and emission band widths, at a scan speed of 60 nm/min. Spectra were corrected for the contribution of light scattering due to the presence of liposomes. Data was exported and analyzed using Microsoft Excel.

Chapter 3 Semi-conserved FGLK domain of the transit peptide involved in the interaction with translocon apparatus

3.1 Abstract

Most chloroplast proteins are encoded as higher molecular weight precursors with an N-terminal extension called the transit peptide, which contains the necessary and sufficient information to target the preproteins back into the chloroplast. A loosely conserved FGLK motif in transit peptides was identified by Pilon and was further investigated bioinformatically. Analysis of the transit peptide of the precursor to the small subunit of Rubisco indicates 2 such motifs: F_{26}TGLK and F_{35}PVSRK. To investigate the role of Phe residue in the two similar domains of SStp and the function of these motifs in the import process, 6 single mutations and 9 double mutants that include F→W, F→S, and F→A at both positions, were generated, expressed and purified from *E. coli.* The effects of these amino acid changes on prSSU import were tested using competitive import inhibition of ^{35}S-labeled wild type (WT)-prSSU. Calculation of IC_{50} indicates that both phenylalanines were important for the import process and different mutations showed different severity in their import ability. Phe →Trp mutants behave most like to the wild type control, the Ala and Ser mutants behave similarly, with IC_{50} closest to that of mSSU. Double mutants showed the most severe loss of competitive ability. Possible mechanisms by which both phenylalanine residues of SStp are involved in the import recognition and binding are discussed.

3.2 Introduction

The large majority of chloroplast proteins are encoded in nuclear genes and synthesized on cytoplasmic 80S ribosomes as larger precursors with an extra peptide extension (Abdallah et al 2000; Canalier-Smith 2000). The extra peptide called a transit peptide is located at the amino terminal end of precursor proteins and is responsible for initiating the import of precursors into chloroplasts by interacting with transport machinery, located in the outer and inner envelope membranes of chloroplasts. It was shown to be both necessary and essential for the import process (Anderson & Smith 1986; Mishkind et al 1985; Van de Broeck et al 1985). However, when the primary sequences of transit peptides from different preproteins are compared, no sequence identity is found (Keegstra et al 1989; von Heijne et al 1989). They are highly heterogeneous in length, composition and organization (Bruce 2001). For a given preprotein, there is significant sequence similarity in the transit peptides among different plant species, whereas little sequence homology is found among different precursor proteins within a given species (Keegstra et al 1989; Pilon et al 1995). Although they have little sequence homology, they do share some common characteristics. Generally, transit peptides are rich in hydroxylated amino acids and hydrophobic residues. They have more basic residues than acidic amino acids, so have a net positive charge (Keegstra et al 1989). The initial dipeptide is almost always methionine-alanine (von Heijne et al 1989).

Common motifs have been identified in different organellar targeting signals. Mitochondrial presequences all share an amphipathic helix, with positively charged amino acids on one side and hydrophobic residues on the other side (Abe et al 2000; Pfanner 2000). However, this feature cannot be easily found in chloroplast transit

peptides. Despite the absence of homology among transit peptide primary sequences, a loosely conserved motif FGLK was identified in 25 precursor proteins (Pilon et al 1995), and for *Silene pratensis* trFd, the only FGLK motif was in the C-terminal domain. 3 domains have been divided in the transit peptide according to deletion studies: the first N-terminal domain mediates the initial binding; the second domain, the middle region of the transit sequence, is involved in envelope translocation after the initial recognition; and the third domain consisting of the C-terminal region is mainly involved in processing (Pilon et al 1995; Rensink et al 1998).

Using the *in vitro* protein import into isolated pea chloroplasts, Pilon et al studied the deletion mutants of trFd, the transit peptide of the precursor to ferredoxin (Pilon et al 1995). They found that partial deletion of the identified semi-conserved FGLK motif (F position remained but was substituted with W) decreased the targeting of preFd to the chloroplast but import still took place. Perry and Reiss did some work on SStp that suggested that the central region that contains both motifs was involved in the binding process (Perry et al 1991; Reiss et al 1989). This observation was further supported by an *in vivo* experiment. Using Green Fluorescent Protein as a probe, the deletion of FGLK motifs was found to disrupt protein targeting to the chloroplast (Lee et al 2002). So somehow, this FGLK motif is involved in preprotein targeting/binding to the chloroplast. As to what role a single amino acid would play in the import process, not much work was done on it yet.

In the current experiments, we used bioinformatics tools to identify and characterize the loosely conserved FGLK motif from different data sets. And based on the results, we have evaluated the import activity of the precursor to the small subunit of Rubisco

(prSSU), the mature domain (mSSU), and thirteen prSSU mutants that have replaced Phe in positions 26 and 35. Using these preproteins as competitors of the radiolabeled precursor ^{35}S-prSSU during import, we demonstrated that phenylalanine in the two FGLK motifs are of great importance for the import efficiency, and this indirectly reflected the involvement of the semiconserved motif in the import process.

3.3 Results

3.3.1 Bioinformatics study of the FGLK motif

With the recent development of genomic databases, it is possible to utilize a large amount of data rather than being limited to single sequence. some bioinformatics study was done by Xinxia Peng using TargetP Predictor on several data sets that contain different organelle-destined preproteins with experimentally determined cleavage sites. The data sets were searched with an amino acid window width of 4-7, and many transit peptides are found to contain a semi-conserved motif, FGLK, which may function as a Toc43 recognition element (Figure 3-1 A). This domain contains one or more of the following elements: a). helix breaking residues that introduce flexibility to the structure, P/G; b). aromatic residue, F/W; c). hydrophobic residues, A/L; d). basic residues, R/K; e) hydroxyl residues, S/T. This non-positional FGLK motif is preferentially found in plastid preprotein transit peptide, and is rare in secreted, nuclear, or cytosolic proteins (Figure 3-1 B).

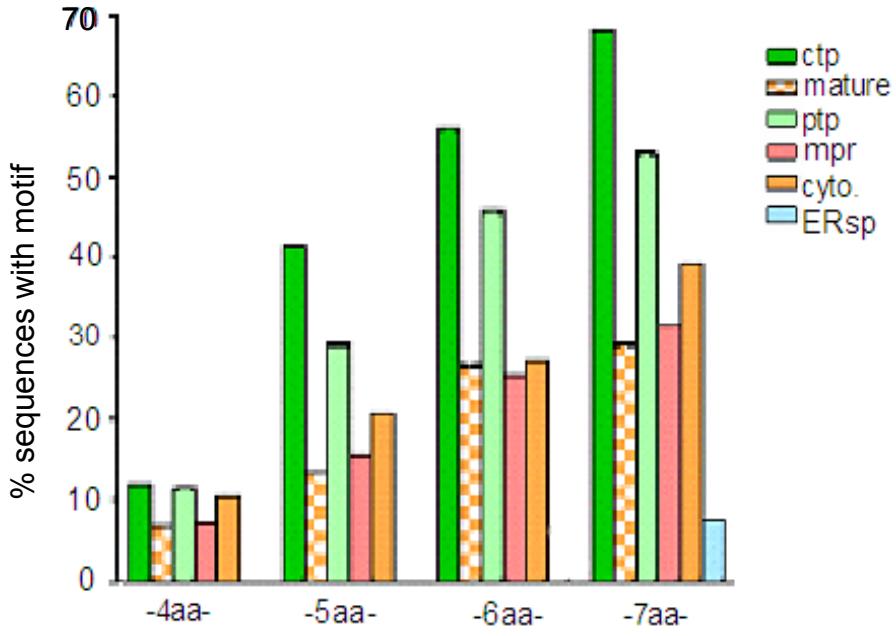
To look at the characteristics of the amino acid composition of each data sets, we then did Anova (Analysis of variance) analysis and compare it to that of chloroplast transit peptide. As is shown in Figure 3-2 A, the amino acid composition varies substantially among

Figure 3-1 Bioinformatic study of the FGLK motif on different organelle-destined preproteins using TargetP data set. The data set contains 141 chloroplast proteins, 368 mitochondria proteins, 269 secreted proteins, 102 nuclear proteins, and 195 cytosolic proteins; chloroplast proteins, mitochondria proteins, and secreted proteins have an experimentally determined cleavage site.

A, The identification of Toc34 recognition motifs. Ctp stands for chloroplast transit peptide; mature means the N-terminal stretch of mature chloroplast proteins that has the same length as the corresponding transit peptide; ptp is the plastid transit peptide; mpr: mitochondrial presequence; cyto: cytosol proteins; ERsp: ER signal peptide.

B, Occurrence frequency of FGLK motif in targeting signals from different organelle-destined preproteins. All data sets were searched with a window of 6 that has the following restriction: F/W and P/G and K/R and A/L and S/T not D/E.

A


70
60
50
40
30
20
10
0
% sequences with motif
-4aa-
-5aa-
-6aa-
-7aa-
ctp
mature
ptp
mpr
cyto.
ERsp

B

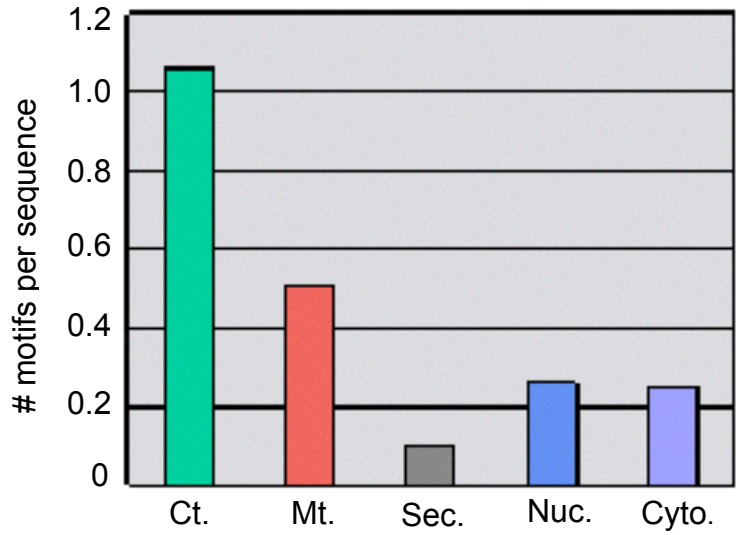
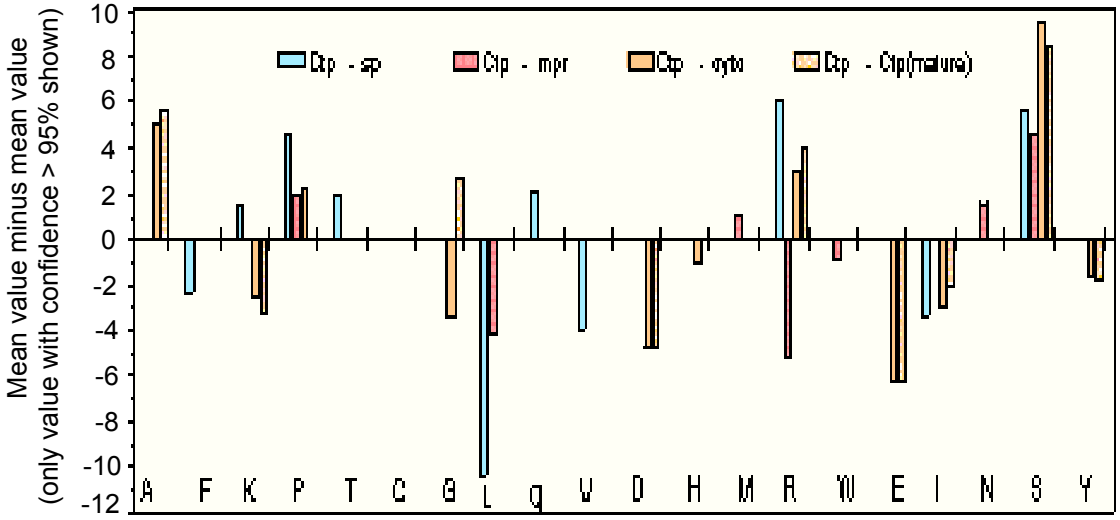

1.2
1.0
0.8
0.6
0.4
0.2
0
motifs per sequence
Ct.
Mt.
Sec.
Nuc.
Cyto.

Figure 3-2 Amino acid composition analysis and Logo plot of amino acids in the loosely conserved FGLK motif. The data base used is Target P.

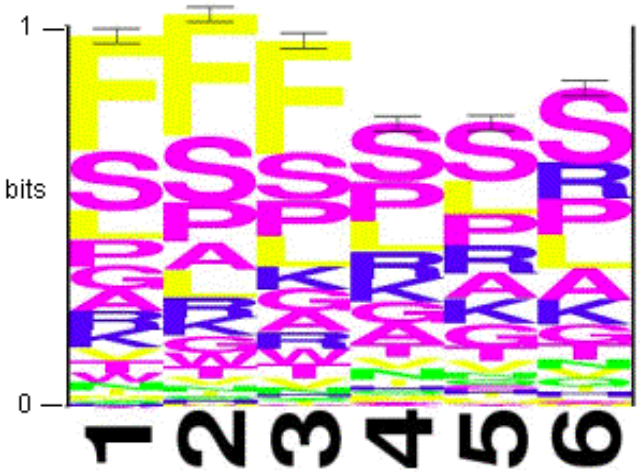
A, Annova analysis of statistically significant differences between amino acid compositions of chloroplast transit peptide (Ctp) and signal peptides (sp), mitochondria presequence (mpr), cytosolic proteins (cyto), and mature chloroplast proteins (mature): the N-terminal stretch of mature chloroplast proteins that has the same length as the corresponding transit peptide. (done by Xinxia Peng)

B, Logo plot of the amino acids in the FGLK motif. All FGLK motifs that are found in the 141 chloroplast proteins are taken out and aligned together to test the frequency of each amino acid. The bigger the font of the letter, the more frequently the amino acid shows up. (done by David McWilliams)

A


Mean value minus mean value
(only value with confidence > 95% shown)
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
A F K P T C G L q V D H M R W E I N S Y

B

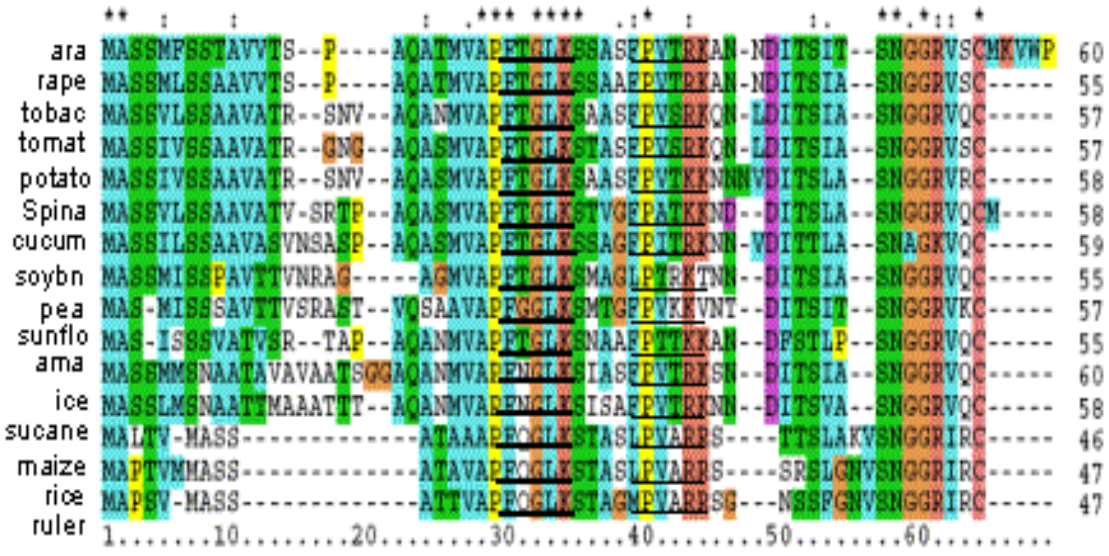

1
bits
0
1 2 3 4 5 6

different data sets. Compared to other proteins, transit peptide has the highest amount of hydroxylated amino acids threonine and serine, especially serine. For positive residues lysine and arginine, mitochondria presequence has equal amount of lysine to that of the transit peptide, but has much higher content of arginine. But on average, there are more arginines in transit peptide than in signal peptide, cytosolic proteins, as well as chloroplast mature proteins that are tested only at the N- terminal stretch, which has equal length to that of the corresponding transit peptide. For aromatic residue phenylalanine, only the signal peptide has more of this residue, others have the same amount as the transit peptide does. In addition, we also can see from the figure that the transit peptide is richer in helix breaking residue proline, but has fewer glycines than cytosol proteins do.

Logo plot (David McWilliams) (Figure 3-2 B) demonstrated that phenylalanine has the highest frequency in the N-terminus as presented by the big font of F letter. The characteristic that transit peptide has high serine content also can be identified from this plot. Serine has a high chance of appearance in each position. Leu, Pro, Arg, Gly and Ala also appear frequently in this FGLK motif. Though bioinformatics studies have identified this motif in a broader range of proteins, its actual function in protein import needs to be further confirmed for confidence.

As transit peptides differ greatly, it is difficult to align them. However, within one precursor species, a good alignment is possible (Keegstra & Olsen 1989). After identification of the FGLK motif on a broader range of proteins, we then looked carefully at a set of prSSU with known cleavage sites and found that there are two FGLK-like motifs in their transit peptides in all the 15 species (Figure 3-3). In the N-terminus, the conserved initial dipeptide MA is present for each of the peptides aligned. The.

Figure 3-3 Alignment of transit peptides of prSSU (SStp). Conserved (*) and semi-conserved (:) (.) residues are indicated. Single letter code is used. Sequences corresponding to the first FGLK motif are underlined with bold lines and those to the second are underlined with thin lines. The sequences used are from Arabidopsis (ara), rape (rape), tobacco (tobac), tomato (tomat), potato (potato), spinach (spina), cucumber (cucum), soybean (soybn), pea, sunflower (sunflo), grain amaranth (ama), ice plant (ice), sugarcane (sucane), maize (maize), rice (rice).


ara
rape
tobac
tomat
potato
Spina
cucum
soybn
pea
sunflo
ama
ice
sucane
maize
rice
ruler
1.....10.....20.....30.....40.....50.....60.....

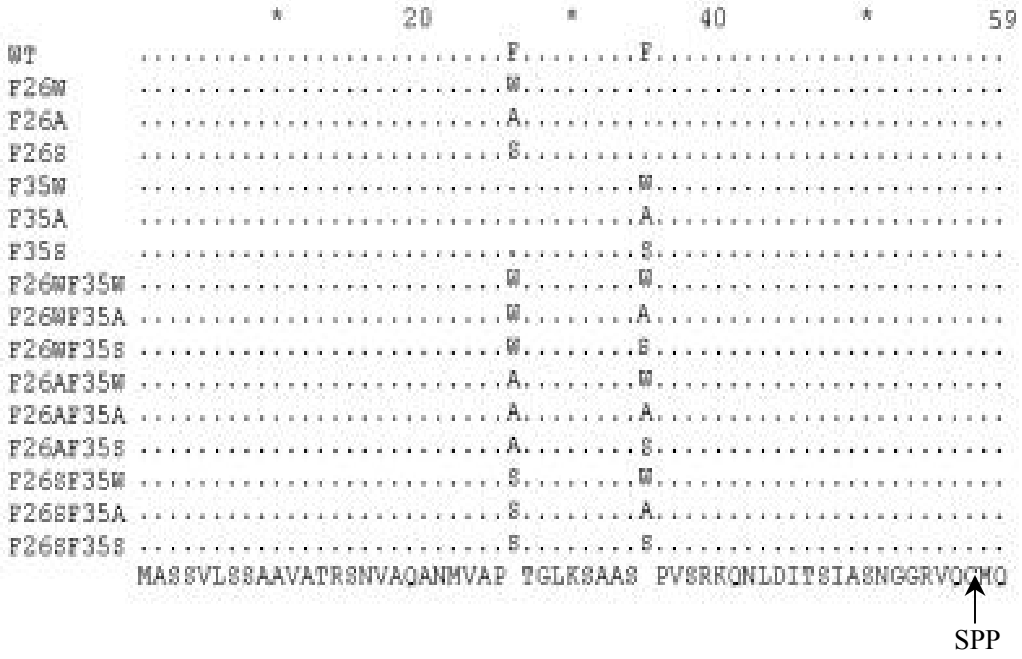
semiconserved processing site motif I/V-X-A/C (Favel & von Heijne 1990) can be easily identified in the C-terminal end. For the middle region, it has most of the highly conserved amino acids. The first of the two FGLK motifs is located in the highly conserved region, while the second one is slightly less conserved as can be seen from Figure 3-3. 4 out of 15 sequences have the hydrophobic residue Leu/Met instead of the aromatic residue in the second motif. Also in this middle region, the helix breaking residues (2 prolines and 1 glycine) are highly conserved through all the sequences, indicating an unusual role of this region.

3.3.2 Generation and purification of F to W/A/S prSSU mutants

To identify the role of this loosely conserved motif FGLK in the preprotein targeting/translocating into chloroplasts, and to determine the effect of the aromatic residue Phe in this motif, several different mutations were made and characterized. Mutation was carried out using the multi-quick mutagenesis kit from Stratagene (La Jolla, CA) with small modifications. Six single strand primers were used in one reaction tube, three for each site instead of one for a particular mutation site (Primer sequences are listed in Table 2-1). Sequencing-confirmed 15 plasmids were replicated in *E. coli* strain XL-blue and extracted using Wizard kit from Promega (Madison, WI). Out of 15 mutants that were generated, 13 were further studied for the competition assay. The mutants that were generated in this study are listed in Figure 3-4.

Wild type (WT), mutants and mature protein were expressed in *E. coli* BL21 [DE3] RIL and a small-scale induction screen was done to select the best colony for expression before scaling the culture up. Proteins used in this study form inclusion

Figure 3-4 Sequence information of prSSU mutants. Only the transit peptide is shown. The amino acid changed is indicated. Identical amino acids are represented by dots. The arrow indicates the position corresponding to the stromal processing peptidase (SPP) processing site.


* 20 * 40 * 59
WT .....F.....F.....
F26W .....W.....
F26A .....A.....
F26S .....S.....
F35W .....W.....
F35A .....A.....
F35S .....S.....
F26WF35W .....W.....W.....
F26WF35A .....W.....A.....
F26WF35S .....W.....S.....
F26AF35W .....A.....W.....
F26AF35A .....A.....A.....
F26AF35S .....A.....S.....
F26SF35W .....S.....W.....
F26SF35A .....S.....A.....
F26SF35S .....S.....S.....
MASSVLSSAAVATRSNVAQANMVAP TGLKSAAS PVSRKQNLDITSIASNGGRVQQMQ
SPP

bodies in bacteria. Proteins were extracted out of inclusion bodies according to the method discussed in Chapter 2. A gel picture was taken to show the purification process (Figure 3-5). In the total cell lysate (shown in lane T), induction with 1 mM IPTG yielded a prominent band with an apparent molecular weight ~20 kD, which is the expected molecular weight of prSSU. Since the proteins expressed were in inclusion bodies, no obvious 20 kD band was detected in the supernatant after centrifugation during the washing and rinsing steps (Figure 3-5 lanes wash and rinse). The final pellet was resuspended in 8 M urea/50 mM DTT. The target protein was in supernatant this time after spin and sample was loaded in lane "8 M urea".

For substitutions of Phe at position 26th, mutants were named as F26W, F26A, F26S and for mutants at position 35th, mutants were named as F35W, F35A and F35S. Double mutants that have the same variation at both positions were named as F26WF35W, F26AF35A and F26SF35S. Hybrid mutation (different mutations for each site) was named accordingly, also. All purified proteins were run on 18% SDS-PAGE for purity analysis. The gel showed that all the proteins were purified to near homogeneity (Figure 3-6).

3.3.3 Import competition and IC_{50} determination for ^{35}S-prSSU, prSSU, mSSU, F26W/A/S and F35W/A/S single mutants

Two FGLK motifs are identified in SStp (Figure 3-3) and the aromatic residue Phe was found to have the highest frequency in this motif (Figure 3-2 B). Mutations were made on the aromatic residue Phe at both motifs to investigate what the influences of the single phenylalanine mutations are on the precursor import into isolated

Figure 3-5 Coomassie stained 15% SDS-PAGE gel of purified F26W-prSSU.

5 μl Bio-Rad broad range marker was loaded in lane "M". The molecular weight of each band is shown along the frame. Sample in lane "T" was the total cell lysate after cells were induced with 1 mM IPTG for 3 hours. The lysate was obtained by tip sonication. Cell pellets were washed 4 times with buffer A/0.1% Triton X-100 and rinsed 3 times with buffer A without Triton. Supernatant after each spin at 40,000 × g was loaded for each step. Protein was finally resolved in 8 M urea with 50 mM DTT which was run in lane "8 M urea".

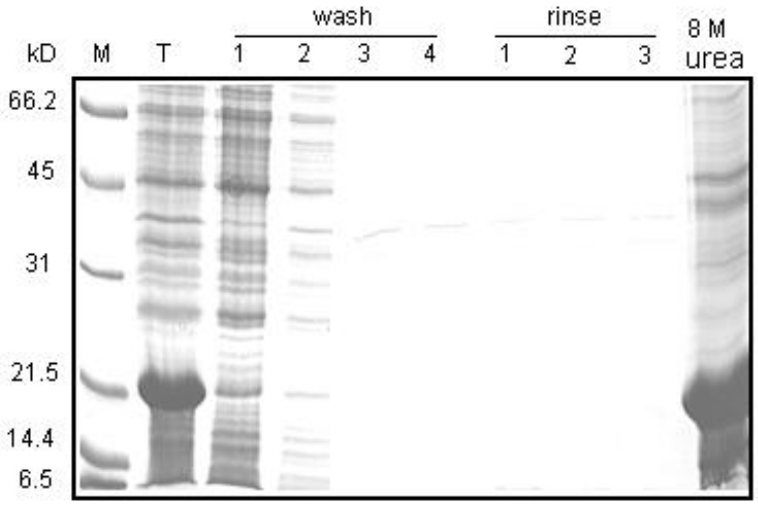

wash
rinse
kD
M
T
1
2
3
4
1
2
3
8 M
urea
66.2
45
31
21.5
14.4
6.5

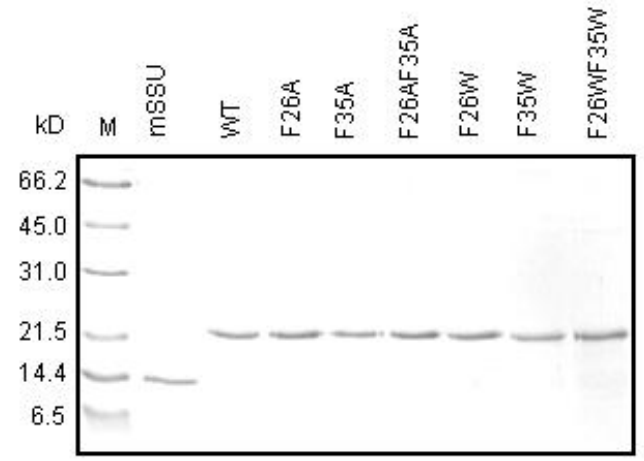
Figure 3-6 Coomassie stained 18% SDS-PAGE gel of all the prSSU mutants.

Proteins were resolved in 8 M Urea/50 mM DTT. 5 μl Bio-Rad broad range marker was loaded in lane "M". mSSU is the mature protein of the small subunit of Rubisco, purified using the same method as all the full-length prSSU proteins.

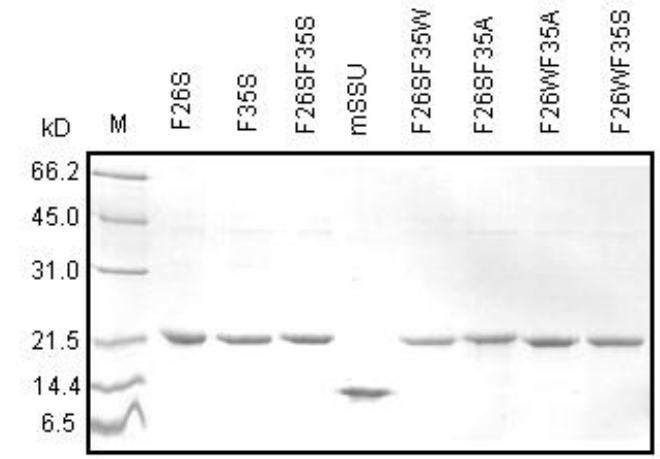
A, mSSU, F26A, F35A, F26AF35A, F26W, F35W, F26WF35W prSSU mutants were shown in that sequence. 1 μg protein was loaded in each lane.

B, F26S, F35S, F26SF35S, mSSU, F26SF35A, F26SF35W, F26WF35A, F26WF35S were run in that order. 2 μg protein was loaded in each lane.

A


kD
M
mSSU
WT
F26A
F35A
F26AF35A
F26W
F35W
F26WF35W
66.2
45.0
31.0
21.5
14.4
6.5

B


kD
M
F26S
F35S
F26SF35S
mSSU
F26SF35W
F26SF35A
F26WF35A
F26WF35S
66.2
45.0
31.0
21.5
14.4
6.5

chloroplasts and thus further deduce the role of this FGLK motif. Purified prSSU, mSSU, F26W/A/S, and F35W/A/S were used as competitors of ^{35}S-prSSU import *in vitro*. In all the import competition assays done in this chapter, the concentration of ^{35}S-prSSU used in the competition assays was fixed at 100 nM, which is well below the *Km* 282 nM (Dabney-Smith et al 1999). The reaction was incubated at room temperature for 20 min, within which the reaction takes places linearly to the substrate concentration. The competitor concentration was set from 0-600 nM. Figure 3-7A shows a filmless autoradiograph image of the import assays using unlabeled mSSU, F35A and wild type prSSU as competitors. The bands were quantified and analyzed as mentioned in Materials and Methods. Figure 3-7 B and C illustrate direct quantification of four independent assays for each protein. B shows the results of the F26 single mutants and C represents the F35 single mutants. All data points were normalized to the control import when there is no competitor present.

Decreasing levels of mSSU accumulation with increasing protein concentration indicates that wild type prSSU is able to inhibit ^{35}S-prSSU import into the chloroplasts (Figure 3-7 A). However, mSSU cannot compete with ^{35}S-prSSU in import into the isolated chloroplasts, even at the highest concentrations tested, which is indicated by the band intensity of the labeled processed prSSU (mSSU) in the image. This suggests that mSSU cannot be recognized and imported into the chloroplast without the presence of the transit peptide. For F35A mutant, less mSSU was accumulated as the concentration was increased. The decrease in the band intensity, however, is very small, suggesting that F35A has partially lost its competitive ability in import into the chloroplast.

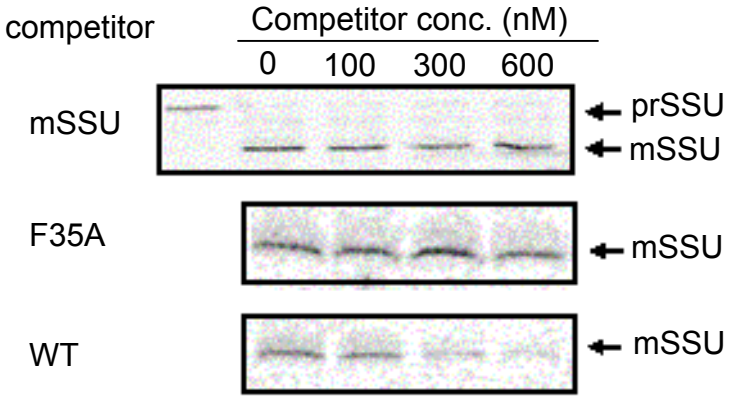
Figure 3-7 Import competitions for prSSU, mSSU, F26 and F35 mutants.

A, SDS-PAGE analysis of import competitions between ^{35}S-prSSU and unlabeled competitors prSSU, mSSU, and F35A-prSSU. The concentration of competitor in each reaction is shown on the top of the figure. The concentration of ^{35}S-prSSU was fixed at 100 nM for all the reactions. Reaction was incubated at room temperature for 20 minutes and was halted by rapid dilution with ice-cold import buffer.

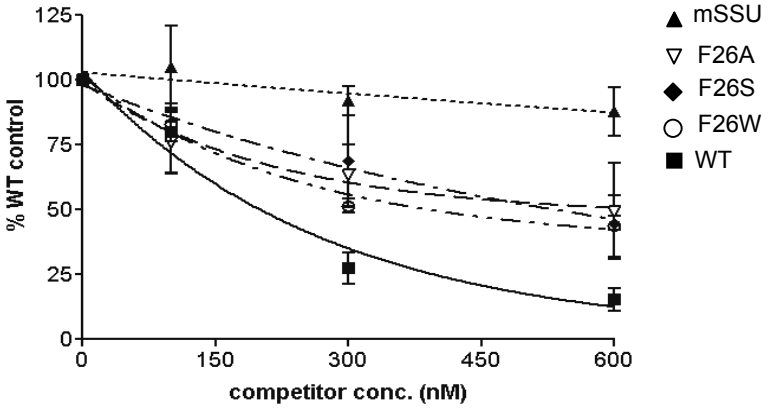
B, Graphical analysis of import competitions between ^{35}S-labeled prSSU and competitors mSSU (▲), F26A (▽), F26S (♦), F26W (o), prSSU (■). Samples were normalized to a no competitor control. All experiments were repeated average 4 times. The data was analyzed as described in the Materials and Methods.

C, Graphical analysis of import competitions between ^{35}S-labeled prSSU and competitors mSSU (▲), F35A (▽), F35S (♦), F35W (o), prSSU (■). Samples were normalized to a no competitor control. All experiments were repeated average 4 times. The data was analyzed as described in the Materials and Methods.

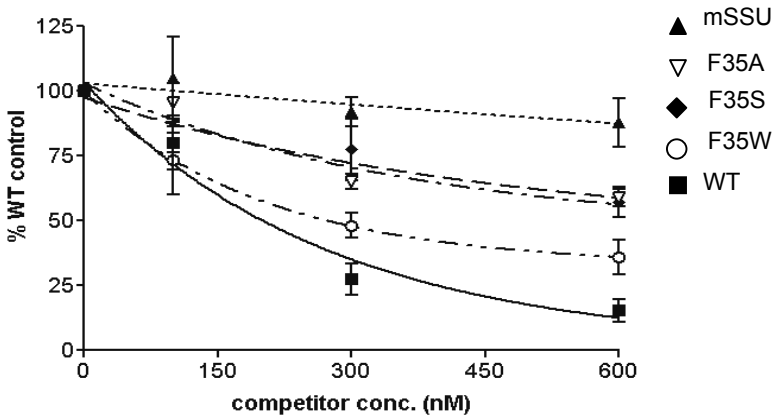
A


competitor
Competitor conc. (nM)
0
100
300
600
mSSU
prSSU
mSSU
F35A
mSSU
WT
mSSU

B


% WT control
125
100
75
50
25
0
0
150
300
450
600
competitor conc. (nM)
mSSU
F26A
F26S
F26W
WT

C


% WT control
125
100
75
50
25
0
0
150
300
450
600
competitor conc. (nM)
mSSU
F35A
F35S
F35W
WT

The inhibition ability of each competitor can be reflected by IC_{50}, which is defined as the concentration of inhibitor required to inhibit 50% of activity. The higher the import competence, the smaller the value of IC_{50}. The IC_{50} for unlabeled prSSU (205 nM) is higher than the theoretical 100 nM; possibly it is due to the contamination of the protein and overestimation of the protein concentration. Trp is a consertive mutation to Phe, both amino acids are bulky and aromatic, so for the Trp mutants at both positions, the calculated IC_{50} is close to the unlabeled wild-type prSSU: F26W 417 nM and F35W 350 nM (Table 3-1).

The behavior of Ser and Ala mutants is more severe than the conservative Trp mutants, as can be seen from Figure 3-7 and the calculated IC_{50}s for F26 mutants: F26A 597 nM; F26S 647 nM. The same trend applies to the mutants at the 35th position. The IC_{50} for F35A is 660 nM and for F35S is 732 nM. When the same Ala and Ser mutation at both sites is compared, the IC_{50} demonstrates that the value of the mutants at the 35th position is lower than that at the 26th position. This demonstrated that the mutants at position 35th show less competitive ability than the mutants at position 26, indicating more involvement of the second original motif in the import process.

3.3.4 Import competitions and IC_{50} determination for Trp, Ala and Ser mutants

Different mutations were made on the aromatic residue Phe in the two FGLK motifs to test if the motifs work cooperatively in the import process. Both single and double mutants were made and tested in the import competition assay. In the experiments illustrated by Figure 3-8 A, purified prSSU, mSSU, Ala single and double mutants were used. Figure 3-8 B demonstrates the behavior of single and double Ser mutants

Table 3-1 Comparison of the import competence of WT and mutant prSSU.

IC_{50} is defined as the concentration of inhibitor required to inhibit 50% of activity. It is calculated by Excel of previously described data.

Competitor	IC_{50} nM
Wild type	205 ± 65
F26WF35W	314 ± 31
F35W	350 ± 73
F26W	417 ± 88
F26A	597 ± 203
F26S	647 ± 134
F35A	660 ± 88
F35S	732 ± 36
F26AF35A	802 ± 150
F26SF35S	1085 ± 205
mSSU	9408 no inhibition

Figure 3-8 Graphical analysis of import competitions between ^{35}S-labeled prSSU and competitors cold prSSU, mSSU, Ala, Ser and Trp mutants.

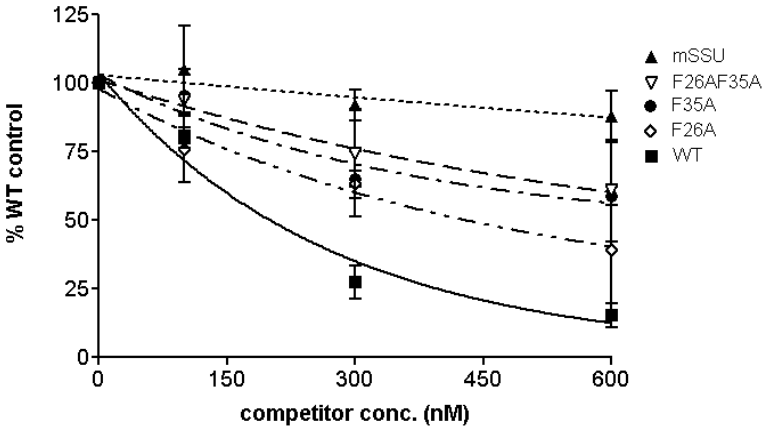
Samples were normalized to a no competitor control. All experiments were repeated average 4 times. The data was analyzed as described in the Materials and Methods.

A, Analysis of import competitions between ^{35}S-labeled prSSU and competitors mSSU (▲), F26AF35A (▽), F35A (●), F26A (◊), prSSU (■).

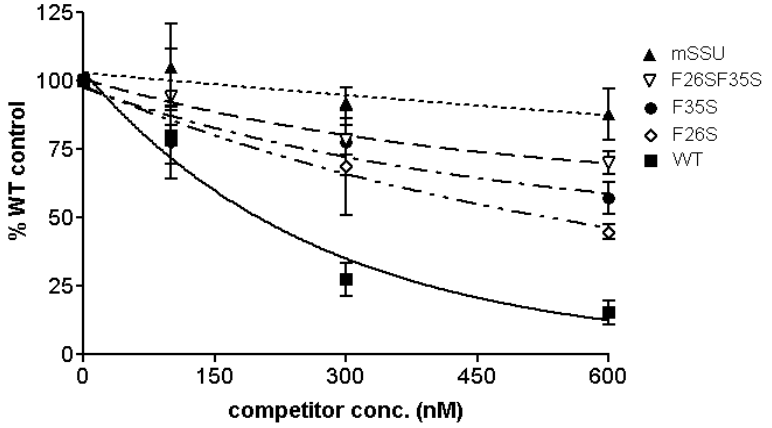
B, Analysis of import competitions between ^{35}S-labeled prSSU and competitors mSSU (▲), F26SF35S (▽), F35S (♦), F26S (◊), prSSU (■).

C, Analysis of import competitions between ^{35}S-labeled prSSU and competitors mSSU (▲), F26W (▽), F35W (♦), F26WF35W(o), prSSU(■).

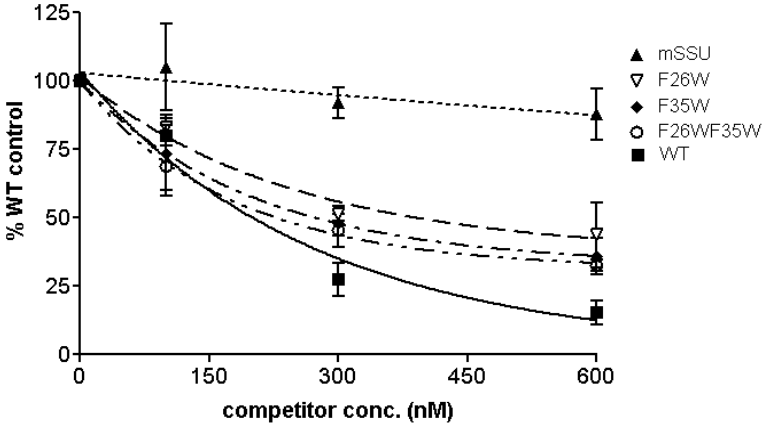
A


% WT control
competitor conc. (nM)
mSSU
F26AF35A
F35A
F26A
WT

B


% WT control
competitor conc. (nM)
mSSU
F26SF35S
F35S
F26S
WT

C


% WT control
competitor conc. (nM)
mSSU
F26W
F35W
F26WF35W
WT

and C illustrates the effects of Trp mutations. The calculated IC_{50} for each mutant is listed in Table 3-1.

Trp mutants behave more like prSSU: they inhibited the import of labeled prSSU to the chloroplasts most effectively (Figure 3-8 C), compared with Ala mutants (Figure 3-8 A) and Ser mutants (Figure 3-8 B). Double Trp mutant (IC_{50} 314 nM) behave closest to WT-prSSU (205 nM), while double Ala (IC_{50} 802 nM) and double Ser mutants (IC_{50} 1085 nM) behave more like mSSU (no inhibition), which means that their ability to compete with prSSU for import is disrupted. The extent of the loss was almost the same for Ser and Ala mutants. Double Trp mutants remain most of WT prSSU's competency in import than any of the two single Trp mutants F26W and F35W; double Ala and Ser mutants are more severely disrupted than any single mutants. However, the effect of the double mutant is not the sum of single mutant, as can tell from the calculated IC_{50} (Table 3-1), suggesting that the two motifs do not act synergistically. Comparing the same substitution at each site, mutations to F35 are more disruptive than equivalent mutation of F26 (Figure 3-8 A, B, C).

3.3.5 Import competitions for prSSU, mSSU and double mutants

Pure (same mutation at both sites) and hybrid (different substitution for each position) double mutants are compared in the import competition assay to investigate if the double mutants follow the same trend as single mutants in their effect of inhibiting import of labeled prSSU. Figure 3-9 A shows that Trp double mutant behaves more like unlabeled wild-type prSSU, while the performance of double Ser and Ala mutants are like the mature protein, which lacks competency for import into chloroplasts. For

Figure 3-9 Graphical analysis of import competitions between ^{35}S-labeled prSSU and competitors cold prSSU, mSSU, pure and hybrid double mutants.

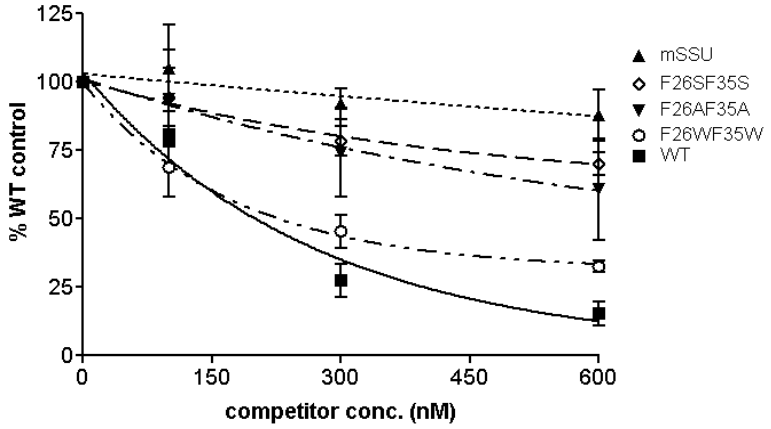
Samples were normalized to a no competitor control. All experiments were repeated average 4 times. The data was analyzed as described in the Materials and Methods.

A, Analysis of import competitions between ^{35}S-labeled prSSU and competitors mSSU (▲), F26SF35S (◊), F26AF35A (▼), F26WF35W (O) and WT (■).

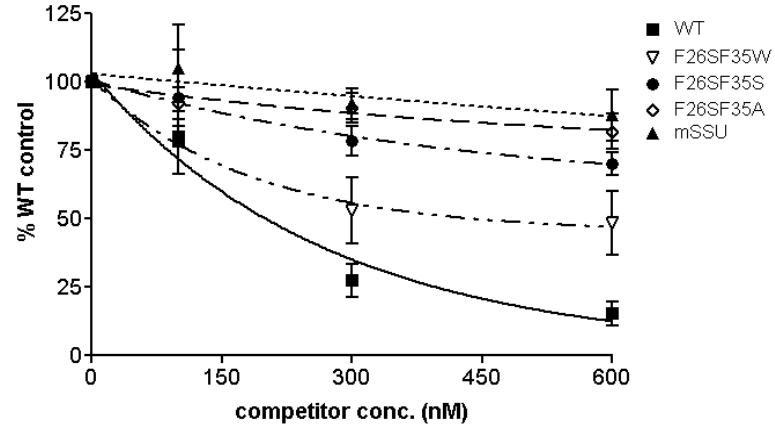
B, Analysis of import competitions between ^{35}S-labeled prSSU and competitors mSSU (▲), F26SF35W (▽), F26SF35S (●), F26SF35A (◊), WT (■).

C, Analysis of import competitions between ^{35}S-labeled prSSU and competitors and mSSU (▲), F26WF35S (◊), F26WF35A (▼), F26WF35W (O), WT (■).

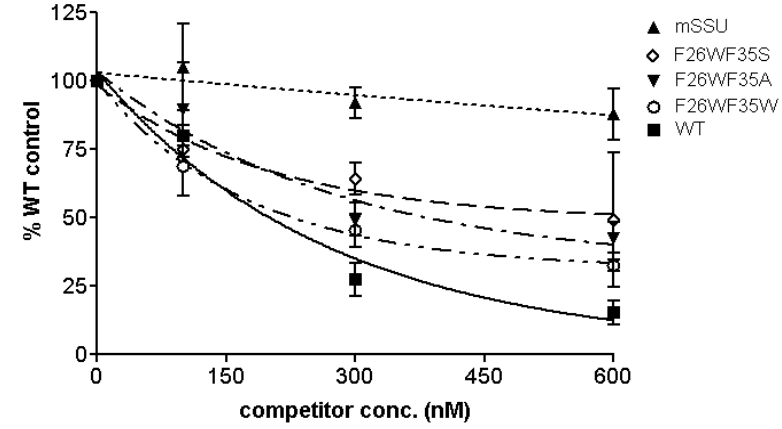
A


125
100
75
50
25
0
% WT control
0
150
300
450
600
competitor conc. (nM)
mSSU
F26SF35S
F26AF35A
F26WF35W
WT

B


125
100
75
50
25
0
% WT control
0
150
300
450
600
competitor conc. (nM)
WT
F26SF35W
F26SF35S
F26SF35A
mSSU

C


125
100
75
50
25
0
% WT control
0
150
300
450
600
competitor conc. (nM)
mSSU
F26WF35S
F26WF35A
F26WF35W
WT

hybrid mutants, if F26 is changed into Trp, while F35 is replaced with Trp, Ser and Ala respectively, the severity is less than those whose Phe at position 26 is changed to Ser, but the same trend is followed as the F35 single mutants (Figure 3-9 B and C). This indicates again that the aromaticity of the amino acid is important for the recognition/binding process.

Both phenylalanines of the two loosely conserved FGLK motifs are involved in the import process, but the role of the two amino acids in the process is not the same. F35 seems to be more involved (Figure 3-8, Figure 3-9). The experiments of hybrid mutants were only repeated twice, IC_{50} was not determined for them. For the pure double mutants, as mentioned in section 3.3.4, the IC_{50} was calculated and listed in table 3-1. For F26WF35W, it was 314 nM, for F26AF35A, the value was 802 nM, and for F26SF35S, the value went to 1085 nM. Serine substitution severely disrupted the function of phenylalanine in the import assay, more severely than the alanine replacement.

3.4 Discussion

3.4.1 Substitution of Phe with Trp, Ser and Ala

Phenylalanine residues at the loosely conserved motifs F_{26}TGLK and F_{35}PVSRK are substituted with tryptophan, serine and alanine to investigate the function of this aromatic residue in the motifs and thus deduce the role of these motifs in the interaction of prSSU with the import apparatus in the chloroplast membranes. Tryptophan and phenylalanine are both aromatic residues and they share similar chemical properties (Teale & Weber 1957), so Trp mutants are more conservative to the wild type. Serine, which has a polar hydroxyl group on the side chain, is the most abundant residue occurring in the transit peptides of chloroplast precursor proteins (10

serines and 3 threonines out of 57 amino acids of SStp, ~22%). Introducing one more might have little effect on the biological activity of the transit peptide. Ala is a small aliphatic residue and is considered to have limited properties as an amino acid (Barrett 1985). The substitution of Phe with Ala abolishes the function of Phe without introducing any other property to the transit peptide. It is a good mutation to study the function of Phe in the import process. An additional property of Trp is that its indole side chain is the most fluorescent of the side chains of amino acids. Since the fluorescence property is highly sensitive to the immediate environment, it is a useful probe for protein structure. This property will be used in the 4th chapter for fluorescence study.

3.4.2 Effect of Phe mutations in the targeting of prSSU to the chloroplast

Early experiments were done on the transit peptides trying to analyze possible function of putative domains. Disruption of the FGLK motif and deletion of some extra C-terminal amino acids of Silene trFd significantly lowered the import efficiency due to decreased targeting (Pilon et al 1995). *In vivo* import experiment utilizing fusion proteins of GFP and deletion mutants of prSSU, also found that the deletion of the FGLK motifs in the central region impaired prSSU's ability to target to the chloroplast (Lee et al 2002). Using a synthetic peptide corresponding to the central region of pea SStp which covered the two loosely conserved FGLK motifs, Perry et al has found that this region is involved in receptor binding (Perry et al 1991). Reiss and his colleagues arrived at the same conclusion from a deletion study in the central regions of SStp (Reiss et al 1989). In addition, the second serine between the two FGLK motifs of SStp was found to be the phosphorylation site and in the binding motif of the 14-3-3 guidance complex (May & Soll 2000), further reinforcing the

importance of the middle region of the transit peptide in the protein import process to the chloroplast.

It is found that the middle region of tobacco SStp contains two semiconserved FGLK motifs and there are 3 helix breaking residues (two prolines and one glycine) inserted in this region. Import competition assays showed that mutation of these three amino acids into alanines has greatly reduced the import efficiency of prSSU (data not shown). This indicates some significance of the structural flexibility of this area, the two motifs might be involved in allowing optimal fitting of recognition motifs.

To further investigate the role of the middle region, we started from the semiconserved FGLK motifs in this study. 13 mutations were made and characterized using import competition assay. The IC_{50} calculated from our experiments indicate the replacement of phenylalanines at the two motifs decreases the affinity of the preprotein for one or more components of the translocation machinery Toc and Tic. And different mutations showed different severity in import ability. Trp mutants have the highest competitive ability among the mutants with only a slight increase in the $IC_{50,}$ behaving more like wide type control. Ala and Ser substitution impaired the activity of prSSU most, as indicated by the calculated IC_{50}. They are much closer to the mSSU negative control, which by itself cannot be recognized and imported by the chloroplast import apparatus. The observation that double Ser and Ala mutants show the most severe loss of inhibition among all the mutants tested, and are similar to the most severe single mutation, suggests the two adjacent motifs do not act cooperatively in the import process.

Actually, the behavior of the Ser mutants surprised us. In the beginning, we thought that since the transit peptides are very enriched in hydroxylated amino acids (about 22% in composition), Ser must have had an important role in the targeting process,

introduction of one more might have not much effect on prSSU's import ability. Ala mutants were proposed to have the severest effect, since Ala is a very dormant residue, not active in the protein reactions (Barrett 1985). However, our results indicate that the introduction of the hydroxyl group is disruptive in this area containing the two loosely conserved FGLK motifs. It even shows more loss of competency than the Ala mutants. Serine is a polar residue, and alanine is hydrophobic though small. Because the replacement of the aromatic residue phenylalanine with the two amino acids impaired prSSU's import competency, aromaticity may be required in this FGLK motif for proper functioning in the import process.

When the activity of these two similar and adjacent FGLK motifs is compared, mutations at position 35th are slightly more severe. This indicates that the Phe at position 35th and the second FGLK motif somehow has a slightly more important role than the first motif that is actually more conserved among the prSSU aligned. In addition, the observation that even the most impaired mutant still imported and processed in the chloroplast, indicates that other parts of the transit peptide or mature domain are also involved in the translocation process.

The two loosely conserved FGLK motifs are located in the middle region of the transit peptide, which was previously suggested to be the linker of the two helices introduced at both ends upon association with membrane. The presence of the helices in a hydrophobic environment was confirmed by NMR work on trFd already (Horniak et al 1993; Wienk et al 1999), and our CD experiment also revealed the presence of helices in SStp (data not shown). The helices are probably more important than the linker region in the recognition/binding/targeting process, and this supports the idea that secondary structure is the recognizable, common motif for the translocon in the double membranes.

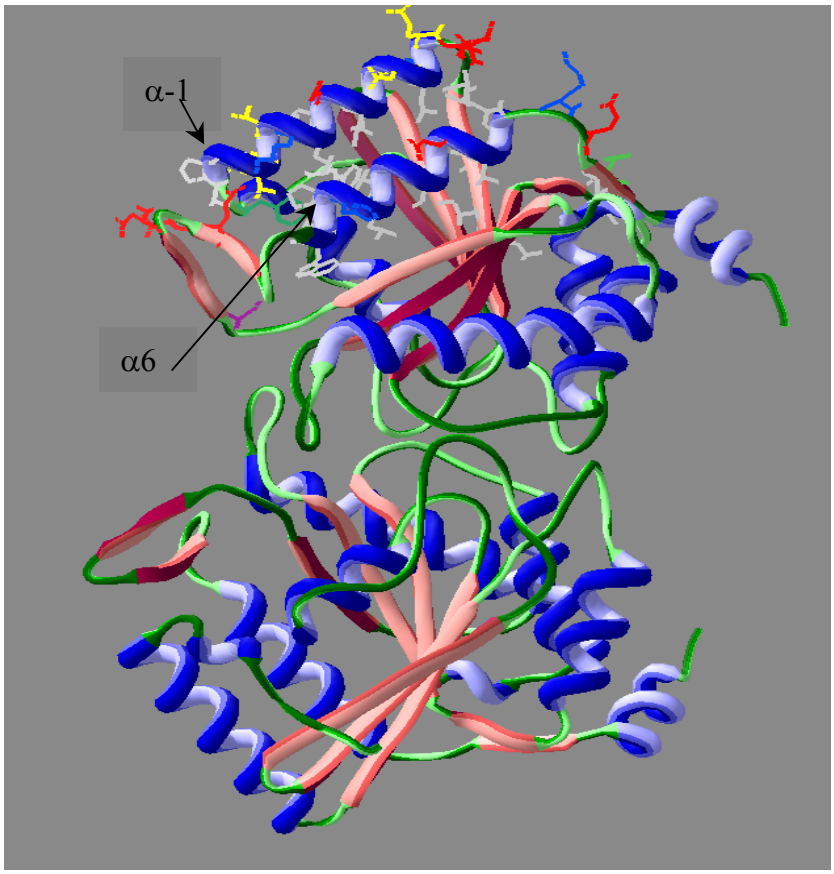
In a summary, the phenylalanine residues and thus the FGLK motifs are important in the prSSU targeting/import process as all mutations interfere with the overall efficiency of import by *in vitro* import competition assay. The reduced activities of the mutant proteins indicate that the interaction between SStp and one or more component of the Toc complex may be driven either by hydrophobic interaction or π-π, π-cation, or π-anion interaction. Although the transit peptide as a group contains high percentage of hydroxylated residues serine and threonine, the introduction of serine into the FGLK motif is not functional, indicating that hydrogen bonding is a minor player involved in the preprotein-import apparatus interaction. As to at which step the motifs interact with the import apparatus, we cannot determine from the experimental evidence above.

We are speculating that the aromatic residues may interact with Toc34, one of the preprotein receptors at the outer envelope membrane during preprotein recognition, possibly via π-π interactions. The phenylalanines in SStp might interact with the aromatic residues in the α-1 and α6 helices of Toc34 facilitating preprotein recognition and binding (Figure 3-10). The positively charged residues arginines and lysines might interact with the acidic amino acids in the two helices too. Also both phenylalanines may possibly be involved in the initial interaction with membrane lipids that will contribute to the import efficiency. We are going to test the interaction of SStp with different artificial membranes in the next chapter.

Figure 3-10 Crystal structure of pea Toc34 dimer

The crystal of Toc34 molecule without the transmembrane domain was obtained in the presence of GTP and Mg^{2+} (not shown in this figure). α-helix is colored in blue, β-sheet is in red and random coil is green for both monomers. For α-1 and α6 helices in monomer 1, the aromatic residues are depicted in grey and dark green; small hydrophobic residues are shown in yellow and acidic residues are in red.

Based on Sun et al, *Nature Struct. Biol.* 2002, 9: 95-100


α-1
α6

Chapter 4 Interaction of SStp with artificial membranes

4.1 Abstract

The transit peptides located at the amino terminal end of chloroplast precursor proteins are responsible for the binding and import of precursors into chloroplasts. Although lacking homology in the primary sequence, common secondary and/or tertiary structure(s) induced upon the association of the preprotein with the chloroplast membranes are postulated to promote recognition of the transit peptide by the transport apparatus located at the envelope membranes. It has been proposed that the requisite structure(s) may be observed only after the transit peptide interacts with the lipids. The interaction of import-competent prSSU proteins (wild type and mutants) with artificial membranes (liposomes) was studied to investigate how precursor-lipid interactions contribute to the import into chloroplasts. Dye-release studies demonstrate that chloroplast precursor proteins interact with chloroplast outer envelope membrane lipids primarily via the transit peptide. This interaction requires the presence of non-bilayer and anionic lipids. prSSU mutants that affected protein import also demonstrated the same trend in their membrane activity, yet the effects were less severe. Fluorescence analysis of the Phe→Trp mutants was used to probe the environment of the central region of transit peptide upon their binding/insertion into artificial bilayers. F26W and F35W mutation indicated a slight difference in their interaction with lipids. Trp at position 26 is located deeper in the membrane and Trp35 is closer to the head group of the lipids, which suggest that they might have different roles in the protein import process to the chloroplast.

4.2 Introduction

Nuclear-encoded, chloroplast-destined proteins have an N-terminal extension that guides the preprotein back into the photosynthetic organelle. Protein trafficking to the chloroplast is a very complicated process. It involves cytosolic targeting factors, molecular chaperones in the cytosol, lipid component and proteinaceous components in the inner and outer envelope membranes, and peptide processing enzymes in the stroma. To unravel the molecular mechanism of translocation, it is essential to understand the interactions between the import machinery and the transit peptide of the precursor. Though they are sufficient and necessary for the complete import of preproteins (Anderson & Smith 1986; Mishkind et al 1985; Van den Broeck et al 1985), the transit peptides are found to share little amino acid sequence homology. A more accepted hypothesis states that interaction between transit peptide and chloroplast envelope membrane lipids induces recognizable, structural motifs that form the common element that can be read by the proteinaceous translocons in the membranes.

The chloroplast outer membrane lipids act not only as a permeability barrier, but also have some function in the biogenesis of the chloroplast. One of their functions may be participation in the protein import process. Several lines of evidence support a role of lipids in chloroplast protein import. Firstly it was shown that preprotein binding and import was greatly impaired in those chloroplasts treated with phospholipase C (Kerber & Soll 1992). Secondly, it was observed that the import of the transit peptide of the precursor to ferredoxin (trFd) still took place after protease treatment of chloroplasts (van't Hof & de Kruijff 1995). Thirdly, if the synthesis of one of the chloroplast membrane lipid components is disturbed by the mutation of the corresponding gene, protein import into chloroplast is also impaired (MGDG, Javis et

al 2000; DGDG, Chen & Li 1998; PG, Müller & Frentzen 2001). Fourth, direct binding of the transit peptide to lipid monolayers or bilayers was observed (Wienk et al 1999; Wienk et al 2000; Pinnaduwage & Bruce 1996; Sabramanian et al 1998). All of these observations support the idea that transit peptide-lipid interaction is involved in the initial binding to chloroplast.

Chloroplast import competition assay done in the earlier chapter showed that both phenylalanines play some role in the targeting/import process. The interaction of purified prSSU proteins (wild type and mutants) with artificial membranes (liposomes) was studied in this chapter to investigate how precursor-lipid interactions contribute to the import into chloroplasts. Using precursor proteins expressed and purified from *E. coli*, we performed dye-release assays to further examine the effect of these site-directed mutations on the interaction with chloroplast outer membrane lipids. Artificial bilayers, with a lipid composition mimicking the outer membrane of chloroplasts, were used to demonstrate that chloroplast precursor proteins do interact with chloroplast outer envelope lipids through a process that is mediated by the transit peptide. Membrane disruption by prSSU proteins needs the presence of non-bilayer and anionic lipids. Electrostatic forces and hydrophobic interactions are involved in the process.

4.2.1 The use of Trp fluorescence

Three native fluorescent amino acids phenylalanine, tyrosine and tryptophan can be used in fluorescence studies. Of the three amino acids, tryptophan is the most popular one because its chromophoric group indole possesses the highest extinction coefficient as well as the highest fluorescence quantum yield. The fluorescence of the indole chromophore is highly sensitive to environment, providing the possibility to

distinguish between hydrophobic and hydrophilic environments and making it an ideal choice for reporting protein conformation changes and interactions with other molecules (Udenfried 1969).

Wild type SStp has no natural tryptophan residue. Thus, to develop a fluorescent reporter, we introduced a single tryptophan at positions 26 and 35 to replace the less fluorescent aromatic amino acid phenylalanine. The replacement of Phe by the larger amino acid Trp slightly decreased its biological function, as was shown by import competition assay using full-length prSSU mutants done in chapter 3. The purpose of this study was to investigate the local conformation of FGLK motifs in SStp upon their association of membranes.

Earlier experiments have shown that preFd inserts efficiently and specifically in lipid monolayers composed of a lipid extract of its target membrane (van't Hof et al 1993) and binds to lipid vesicle (van't Hof & de Kruijff 1995), via its transit peptide. The same has been observed for transit peptide of the precursor to the small subunit of Rubisco, SStp (Pinnawudage & Bruce 1996; Subramanian et al 2001). All these demonstrated that there is an interaction existing between the transit peptides and membrane lipids. This makes the peptide much smaller than the full-length precursor, a suitable molecule to study the structural behavior.

4.3 Results

4.3.1 Dye release assay for prSSU, mSSU and single mutants

It has been shown previously that there is an interaction between membrane lipids and chloroplast transit peptides (van't Hof et al 1993; Pinnaduwage & Bruce 1996; Subramanian et al 2001). To examine that if peptide-membrane interactions were affected by the substitutions made in chapter 3, we carried out dye release assays. OM

liposomes (MG/DG/PC/PG with a molar ratio 20/30/40/10) mimicking the outer envelope membrane of chloroplast and PC liposomes composed of PC alone were made using a bath sonicator, and calcein was enclosed in the small unilamellar vesicles. The dye calcein is self-quenched at concentration above ~50 mM, as is found inside the intact liposomes when protein or Triton X-100 is absent, yet if it is released, it regains it's fluorescence at ~ 517 nm upon dilution. If the protein interacts with liposomes, which mimic the outer envelope membrane of the chloroplast, disruption/penetration of the membrane occurs causing the dye to be released to the bulky solution. The rapid dilution of the dye then can be followed by measuring fluorescence. Increase in fluorescence, due to dye released as a result of protein interaction with the liposome membrane, is normalized to a Triton X-100 control which represents 100% dye release due to total disruption of liposome membrane.

It is shown in Figure 4-1A and 4-1B that all the precursor proteins used were able to induce dye release from OM liposomes mimicking the outer envelope membrane. Increase of protein concentration caused more dye release, indicative of an interaction of proteins with the OM membrane. Wild type prSSU induced ~60% dye release compared to the Triton X-100 control. For the mutated prSSUs, Ala and Ser single mutants at positions 26th and 35th behaved similarly, all caused about 50% dye release, indicating that replacement of aromatic residues with non-aromatic residues decreased the membrane activity. F26W mutant caused a little decrease in the dye release, but it still remained higher activity than Ala and Ser mutants. An exception was observed for F35W mutant that showed slightly higher effect compared to the wild type prSSU. This is probably due to the underestimation of its concentration. For the mature protein mSSU without the transit peptide, only about 20% dye was released. However, none of these import-competent proteins were able to induce dye

Figure 4-1 Dye release assay for WT, mSSU, F26 and F35 mutants.

Proteins were incubated for an hour at room temperature with lipid vesicles to induce dye release. Interactions with OM liposomes mimicking the chloroplast outer envelope membrane (OM, MGDG/DGDG/PC/PG: 20/30/40/10 molar ratio) are illustrated in panels A and B. Interactions with liposomes composed of PC alone are represented in panels C and D. Protein concentrations were varied from 0.0125 to 0.5 μM. All dye release for each protein is compared to the Trition X-100 control.

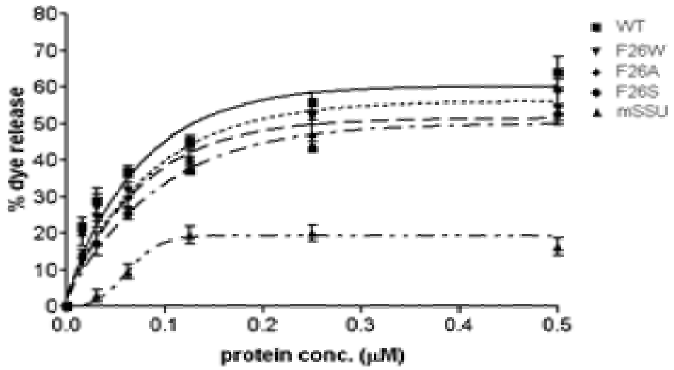
A, Percent dye release plotted against protein concentration for WT (■), F26W (▼), F26A (♦), F26S (●) and mSSU (▲).

B, Percent dye release plotted against protein concentration for WT (■), F35W (▼), F35A (♦), F35S (●) and mSSU (▲).

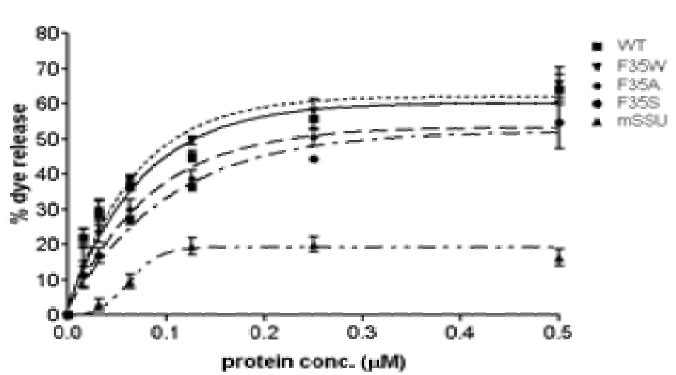
C, Percent dye release plotted against protein concentration for WT (■), F26W (▲), F26A (▼), F26S (♦) and mSSU (●).

D, Percent dye release plotted against protein concentration for WT (■), F35W (▲), F35A (▼), F35 (♦) and mSSU (●).

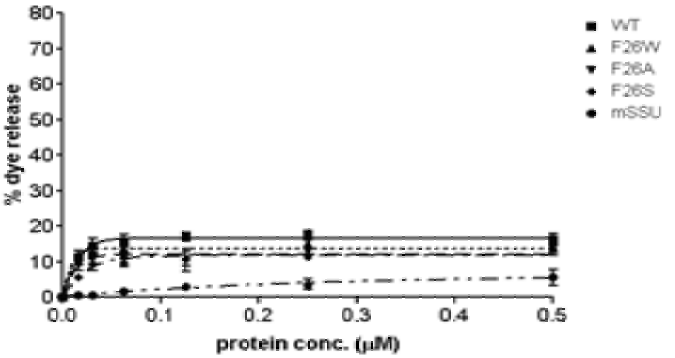
A


F26 mutants + OM
% dye release
protein conc. (μM)
WT
F26W
F26A
F26S
mSSU

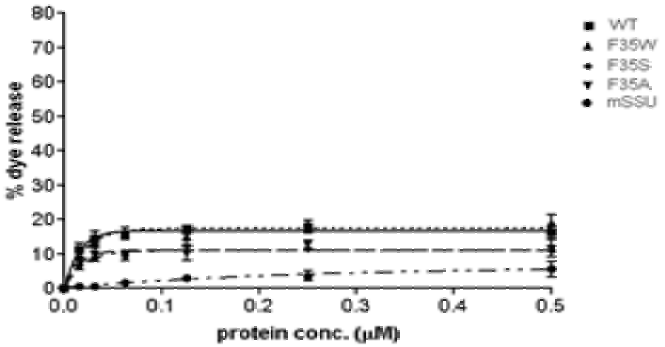
B


F35 mutants + OM
% dye release
protein conc. (μM)
WT
F35W
F35A
F35S
mSSU

C


F26 mutants + PC
% dye release
protein conc. (μM)
WT
F26W
F26A
F26S
mSSU

D


F35 mutants + PC
% dye release
protein conc. (μM)
WT
F35W
F35S
F35A
mSSU

release from the liposomes made of neutral lipid PC. Wild type prSSU shows the maximal membrane activity toward the PC liposomes, only causing about 20% calcein release of the Triton control. This suggests that there is no interaction between the import-competent proteins with neutral phosphocholine lipids (Figure 4-1C and 4-1D).

4.3.2 Dye release assay for prSSU, mSSU, Ala and Ser mutants

Ala and Ser substitution of Phes at both 26th and 35th positions severely impaired the import of prSSU into isolated chloroplasts as shown in Chapter 3. To investigate if their interaction with membrane lipids was affected, dye release assays were performed using the liposomes comprised of lipids mimicking the outer membrane of chloroplasts. Figure 4-2 A and 4-2 B show that as protein concentration increased, dye release from OM liposomes also increased, resulting in ~60% dye release for wild type prSSU relative to the Triton X-100 control. The mature domain alone was not able to induce much dye release, ~20% of control was oberserved. All the Ala and Ser mutants behaved similarly in this assay. They were able to induce dye release to levels comparable to that of the wile type prSSU, only a little decrease was observed. The decrease, however, is not as great as that of the import competency, as revealed by IC_{50} listed in Table 3-1. By contrast to the observation for OM liposomes, these mutants were not able to induce dye release from PC liposomes (panels C and D in Figure 4-2).

Figure 4-2 Dye release assay for WT, mSSU, Ala and Ser mutants.

Proteins were incubated for an hour at room temperature with lipid vesicles to induce dye release. Interactions with OM liposomes mimicking the chloroplast outer envelope membrane (OM, MGDG/DGDG/PC/PG: 20/30/40/10 molar ratio) are illustrated in panels A and B. Interactions with liposomes composed of PC alone are represented in panels C and D. Protein concentrations were varied from 0.0125 to 0.5 μM. All dye release for each protein is compared to the Trition X-100 control.

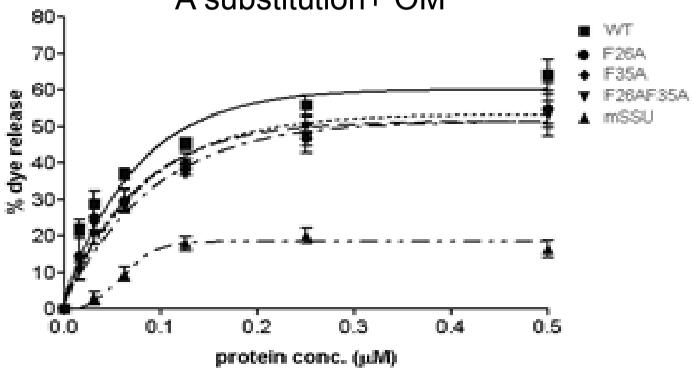
A, Percent dye release plotted against protein concentration for WT (■), F26A (●), F35A (♦), F26AF35A (▼), and mSSU (▲).

B, Percent dye release plotted against protein concentration for WT (■), F35S (♦), F26SF35S (●), F26S (▼), and mSSU (▲).

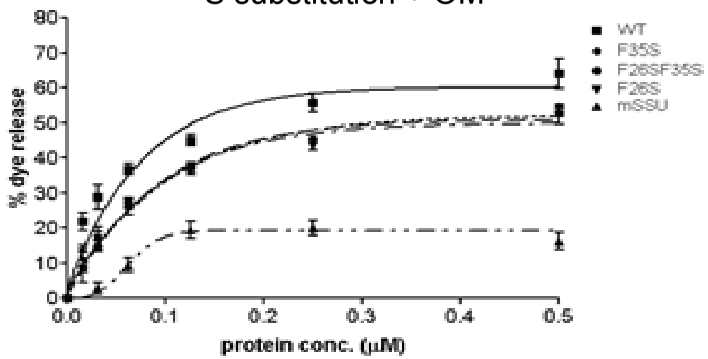
C, Percent dye release plotted against protein concentration for WT (■), F26A (▲), F35A (▼), F26AF35A (♦) and mSSU (●).

D, Percent dye release plotted against protein concentration for WT (■), F35S (▲), F35S (▼), F26SF35S (♦), and mSSU (●).

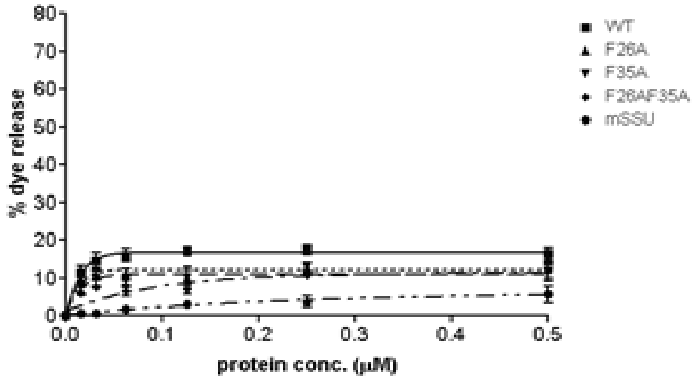
A


A substitution+ OM
% dye release
protein conc. (μM)
WT
F26A
F35A
F26AF35A
mSSU

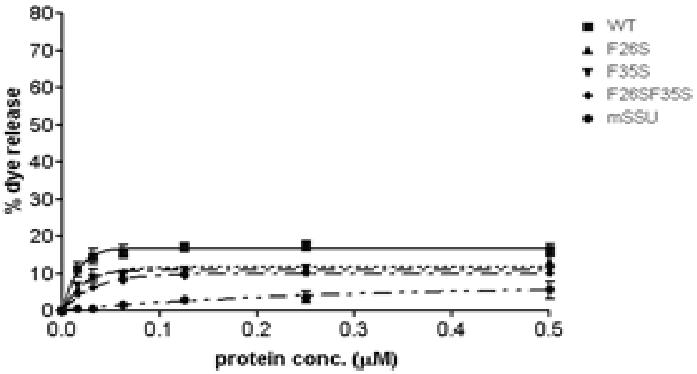
B


S substitution + OM
% dye release
protein conc. (μM)
WT
F35S
F26SF35S
F26S
mSSU

C


A substitution+ PC
% dye release
protein conc. (μM)
WT
F26A
F35A
F26AF35A
mSSU

D


S substitution + PC
% dye release
protein conc. (μM)
WT
F26S
F35S
F26SF35S
mSSU

4.3.3 Dye release assay for prSSU, mSSU, Trp and double mutants

Tryptophan mutants behaved more like wild type prSSU in the import competition assay. To investigate if the interaction with lipid was influenced by the conservative mutation of Phe to Trp, membrane disruption assay was carried out using OM liposomes which mimics the outer membrane of chloroplasts. Figure 4-3 A shows that the Trp mutants still behaved more like the wild type prSSU, among which there was a slight increase for the F35W mutants, while the other two still have less activity though very close to wild type prSSU. For PC liposomes that lacked galactolipids (MGDG and DGDG) and anionic lipid PG, little/no liposome disruption was observed, only less than 20% dye release could be detected (Figure 4-3 C). For the double mutants, similar to the double mutants in the import competition assay, double Trp mutants still functioned most closely to wild type prSSU (Figure 4-3 B). Double Ala and Ser mutants behaved exactly the same in the dye release assay, as the membrane activity of both was decreased slightly (Figure 4-3 B). Again, for liposomes comprised only of neutral lipid PC, little dye release was observed (Figure 4-3 D).

4.3.4 Generation of mutant SStp-pTYB2 constructs

The transit peptide of prSSU (SStp) has already been shown to function properly without the presence of mature protein. It not only can interact directly with the outer membrane lipids (Pilon et al 1995; Pinnaduwage & Bruce 1996; Subramanian et al 2001), but also can be properly imported into isolated chloroplasts, regardless of whether it was chemically synthesized (Perry et al 1991) or isolated from *E. coli* (Pinnaduwage & Bruce 1996; Dabney-Smith et al 1999; Subramanian et al 2001).

Figure 4-3 Dye release assay for WT, mSSU, Trp and double mutants.

Proteins were incubated for an hour at room temperature with lipid vesicles to induce dye release. Interactions with OM liposomes mimicking the chloroplast outer envelope membrane (OM, MGDG/DGDG/PC/PG: 20/30/40/10 molar ratio) are illustrated in panels A and B. Interactions with liposomes composed of PC alone are represented in panels C and D. Protein concentrations were varied from 0.0125 to 0.5 μM. All dye release for each protein is compared to the Trition X-100 control.

A, Percent dye release plotted against protein concentration for WT (■), F35W (♦), F26WF35W (●), F26W (▼), and mSSU (▲).

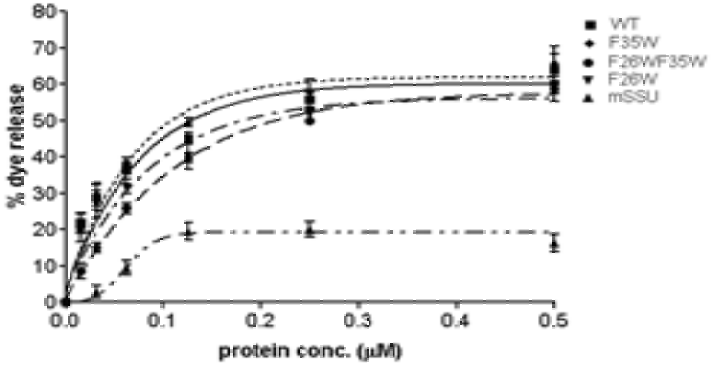
B, Percent dye release plotted against protein concentration for WT (■), F26WF35W (▼), F35AF35A (♦), F26SF35S (●), and mSSU (▲).

C, Percent dye release plotted against protein concentration for WT (■), F26W (▲), F35W (▼), F26WF35W (♦), and mSSU (●).

D, Percent dye release plotted against protein concentration for WT (■), F35WF35W (▲), F26SF35S (▼), F26AF35A (♦), and mSSU (●).

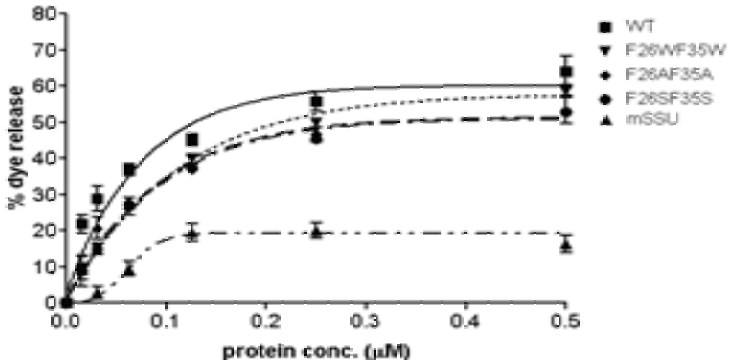
A

W substitution+ OM



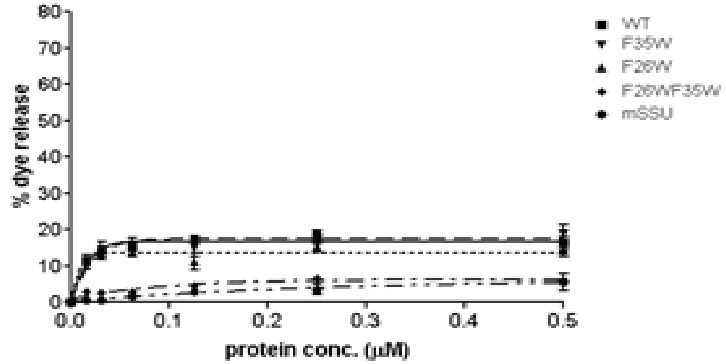
B

double substitution + OM



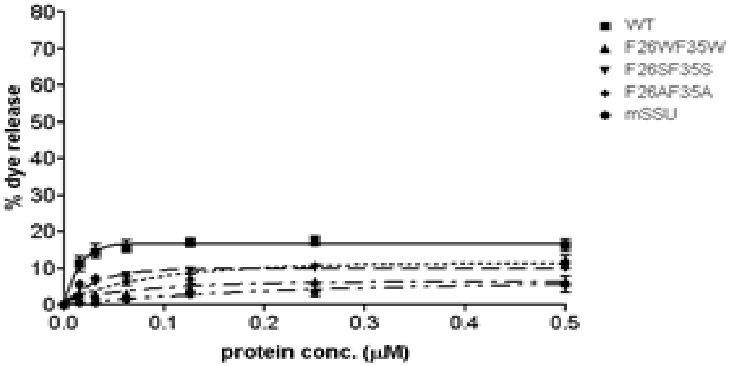
C

W substitution+ PC



D

double substitution + PC



Because of the small size and full functionality, it is much more suitable for biophysical studies.

Interested in the loosely conserved FGLK motifs of SStp, we decided to use mutant SStp to investigate its function. To get the mutant SStp, full length prSSU mutants in the plasmid pET11d generated in Chapter 3 were used as templates for the PCR reaction. After PCR amplification, both SStp mutant and pTYB2 plasmid were double digested by the restriction enzymes Xma I and Nde I. Digested product were gel purified and ligated together. The subcloning strategy used to fuse SStp to the N terminus of intein is outlined in Figure 4-4.

PCR amplification products were run on 6% acrylamide gel (Figure 4-5). All PCR reactions worked as is shown, and the desired fragment of 177 bp was amplified for each mutant. Direct colony PCR screening was carried out to identify positive colonies for ligation products (Figure 4-6). The screening PCR primers used were T7 universal primer and intein reverse primer which located ahead of and behind the multicloning sites respectively. In the event that a PCR fragment of ~222 bp is obtained, this means that the vector is self-ligated after restriction digestion and there is no insert in the plasmid. If the SStp gene is inserted into the pTYB2 vector, the desirable size of the PCR fragment should be 349 bp, as can be seen from most of the colonies we screened. Different SStp mutant inserts were screened as are labeled in the Figure 4-6. Positive clones were grown up, plasmids were extracted, and sequencing was performed for further confirmation.

Figure 4-4 Subcloning strategy of mutant SStp-pTYB2 plasmids.

Mutant SStp was PCR amplified from pET11d plasmids containing mutant full-length prSSU. An Nde I site was inserted at the N-terminal end and an Xma I site was introduced at the C-terminal end. pTYB2 vectors were linearized at the same sites as mutant SStp. Then both digested vector and insert were gel purified and ligated together.

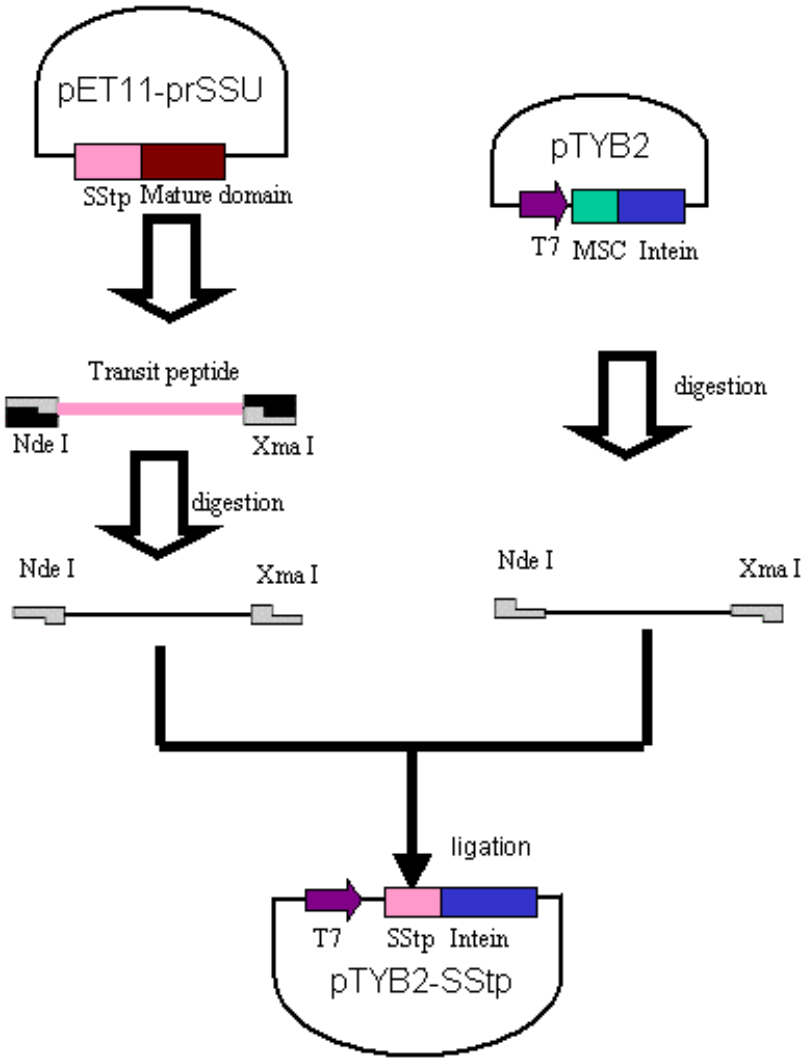

pET11-prSSU
SStp Mature domain
pTYB2
T7 MSC Intein
Transit peptide
Nde I
Xma I
digestion
digestion
Nde I
Xma I
Nde I
Xma I
ligation
T7
SStp Intein
pTYB2-SStp

Figure 4-5 PCR amplification of mutant SStp from plasmid pET11d-prSSU.

Part of the 100bp marker is shown. The fragment size is 177 bp. PCR products were run in 6% acrylamide gel. Lane (-) is a negative control for PCR reaction. Each amplified fragment from different template was run as labeled. 6 μl PCR product was loaded in each lane.

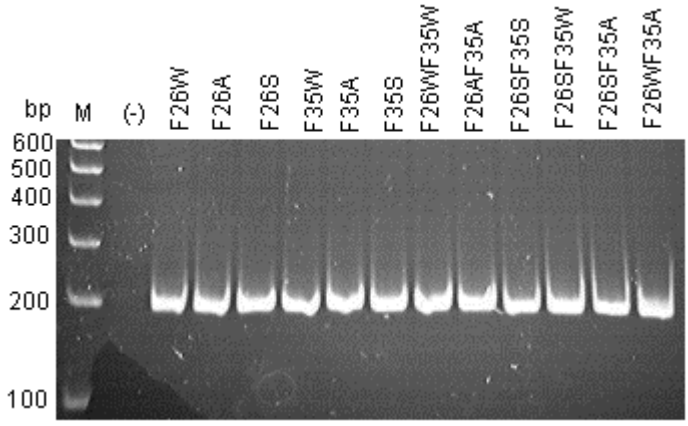

bp
M
(-)
F26W
F26A
F26S
F35W
F35A
F35S
F26WF35W
F26AF35A
F26SF35S
F26SF35W
F26SF35A
F26WF35A
600
500
400
300
200
100

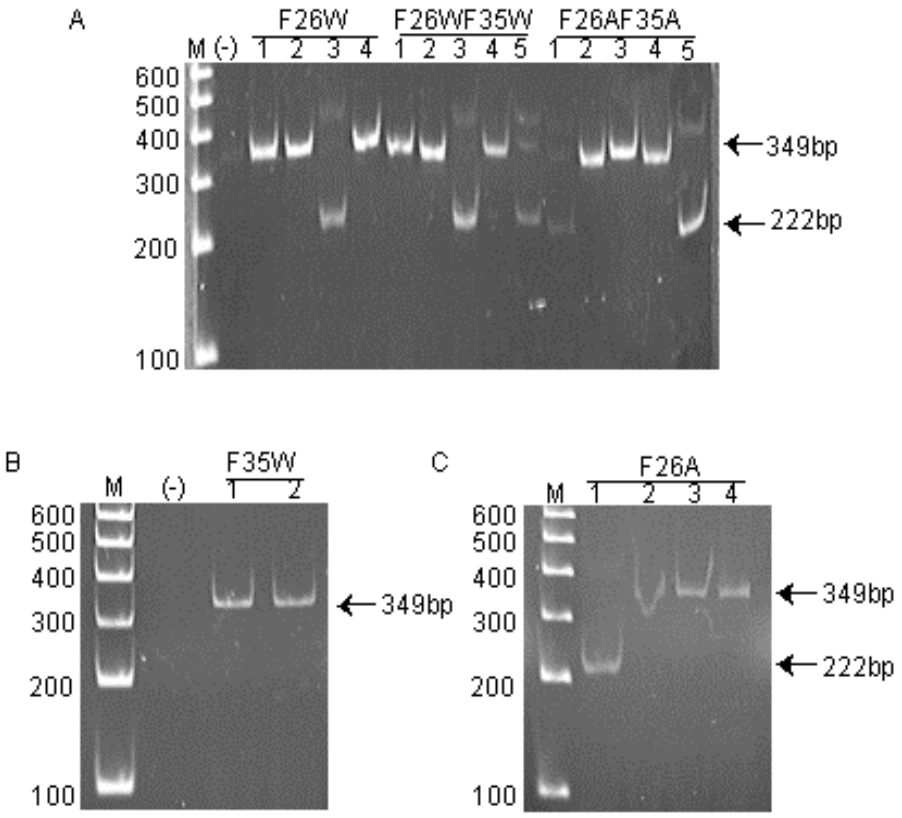
Figure 4-6 Direct colony PCR screening of mutant SStp-pTYB2 transformants.

Part of the 100bp marker is shown and lane (-) is a negative control for PCR reaction. PCR products were run in 6% acrylamide gel. The desirable length of PCR product is 349bp, which indicates the insertion of peptide into pTYB2 vectors. While smaller fragment 222bp indicates that the vector was cut but self-ligated.

A, 4 colonies of F26W-SStp-pTYB2, 5 of F26WF35W-SStp-pTYB2 and 5 of F26AF35A-SStp-pTYB2 are screened.

B, 2 colonies for F35W-SStp-pTYB2 are screened.

C, 4 colonies are screened for positive F26A-SStp-pTYB2 chimeras.


A
F26W
F26WF35W
F26AF35A
M (-) 1 2 3 4 1 2 3 4 5 1 2 3 4 5
600
500
400
300
200
100
349bp
222bp
B
M (-) F35W 1 2
600
500
400
300
200
100
349bp
C
F26A
M 1 2 3 4
600
500
400
300
200
100
349bp
222bp

4.3.5 Expression and purification of SStp mutants

The IMPACT system is a novel protein purification system that utilizes the inducible self-cleavage activity of a protein splicing element (Intein) to separate native SStp from the chitin binding domain (CBD, affinity tag) without the use of an exogenous protease. In our construct, the tag is fused to the C terminus of the target SStp. Upon induction with 1 mM IPTG, the fusion protein N'-SStp-Intein -CBD -C' is expressed in *E. coli* strain ER 2566. The CBD tag allows the affinity purification of the fusion protein on a chitin column. In the presence of thiols like DTT or β-mercaptoethanol or cysteine, the intein undergoes specific self-cleavage that releases native SStp from the chitin-bound intein tag. The schematic diagram for this IMPACT-CN purification system is represented in Figure 2-2.

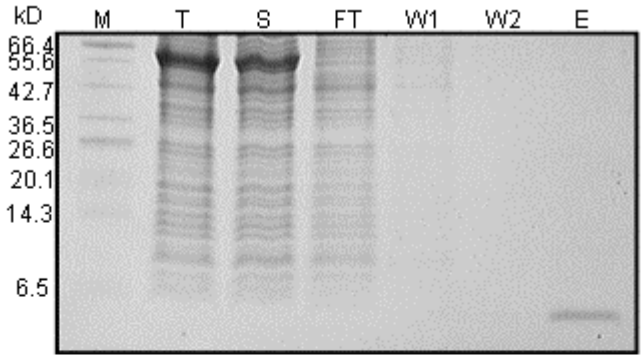
Sequence-confirmed plasmids that contained mutant SStp were transformed into competent *E. coli* strain ER 2566. A small scale induction screen was first performed to select the colony that had the best expression of SStp-Intein-CBD. Then the culture was scaled up to get a large amount of proteins that were purified using the IMPACT system. The purification profile of F26W-SStp is shown in Figure 4-7 A and all protein samples were loaded with non-reducing 4 × SSB (sample solving buffer). From the gel we can tell that induction with 1 mM IPTG yielded a prominent band with an apparent molecular mass of ~63 kD in the total cell lysate. This mobility agrees well with the predicted molecular weight of the fusion SStp-Intein-CBD protein of 62.9 kD. A 63 kD band was evident in the supernatant after cell disruption using French press and sonication followed by centrifugation at 20, 000 × g (Figure 4-7 A lane T), indicating that the expressed protein was in a soluble form. Later, SStp purification was performed using a single chitin column. This affinity matrix was

Figure 4-7 Coomassie stained 19.2% Tris/Tricine gel of SStp mutants.

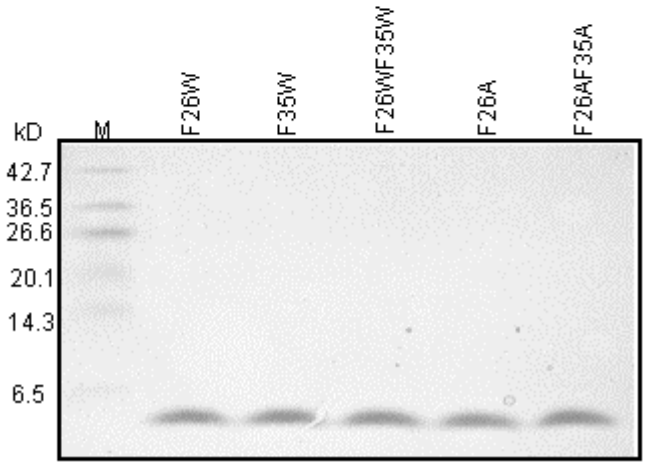
A, purification profile of F26W-SStp from *E. coli*. M stands for the broad range marker (NEB). Only part of the marker is shown and the molecular weights are as specified. T stands for the total cell lysate after the cells were induced with 1 mM IPTG for 3 hours. The lysate was obtained by French pressing the induced cells followed by sonicaion. S is the supernatant obtained after centrifugation of the cell lysate at 20, 000 × g. FT is the flow though from loading the supernatant on chitin column. W1 and W2 are the washes obtained by washing the column using wash buffer. E is the eluted fraction after β-ME cleavage.

B, Electrophoresis of all purified mutant SStp as specified. Purification procedure is the same for all proteins as for F26W-SStp. 2 μg protein/lane were loaded for each protein.

A


kD
M
T
S
FT
W1
W2
E
66.4
55.6
42.7
36.5
26.6
20.1
14.3
6.5

B


kD
M
F26W
F35W
F26WF35W
F26A
F26AF35A
42.7
36.5
26.6
20.1
14.3
6.5

effective in capturing CBD-containing proteins since very little of 63 kD protein was observed in the flow through (Figure 4-7 A lane ft). Binding of the fusion protein to the chitin column was very tight since no 63 kD protein was washed off (Figure 4-7 A Wash 1 & 2). The fusion protein was then incubated overnight with elution buffer that contained 50 mM β-ME which is responsible for starting intein-mediated cleavage. Eluted F26W-SStp is shown in lane E. Another elution may be applied after the first one. Finally all eluted proteins were pooled and lyophilized. F35W-SStp, F26A-SStp, F26WF35W-SStp and F25AF35A-SStp were all purified using the same method and were run in a 19.2% Tris/Tricine gel (Figure 4-7 B)

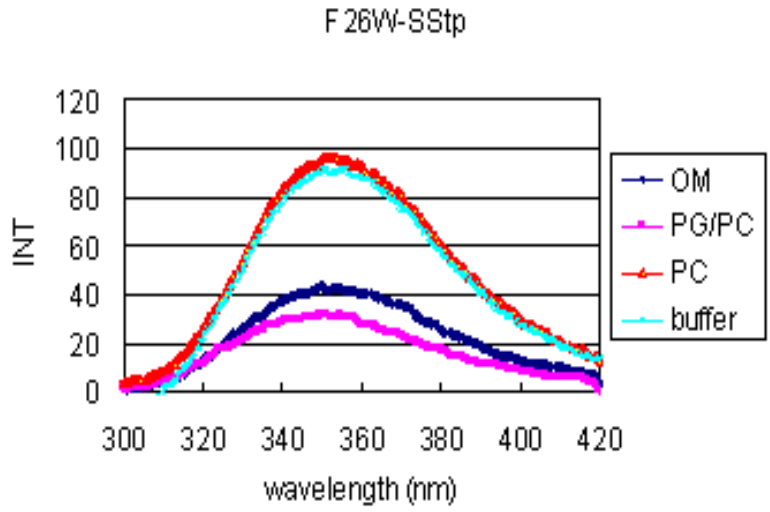
4.3.6 Partitioning of the transit peptide into lipid vesicles

Peptide binding to lipid vesicles was investigated by intrinsic Trp fluorescence emission measurements using F26W-SStp and F35W-SStp. Transit peptides were incubated with lipid vesicles consisting of MGDG/DGDG/PC/PG (20/30/40/10); PC/PG (60/40); or pure PC. The maximum Trp fluorescence emission spectra of the two mutants, measured either in 50 mM Hepes buffer (pH 7.6) or in the presence of lipid vesicles, is shown in Figure 4-8. The maximal emission wavelength for F26W-SStp is ~355 nm in 50 mM Hepes buffer, while it is ~351 nm for F35W-SStp (Table 4-1). The difference of the maximum fluorescence in buffer may reflect the different microenvironments the two Trps are in. Addition of PC vesicles did not affect the shape of the Trp fluorescence spectrum and the Trp fluorescence intensity

Figure 4-8 Fluorescence emission spectra of SStp Trp mutants.

Spectra of SStp Trp mutant in buffer (×), in the presence of liposomes made of PC alone (▲), PC/PG (60/40) liposomes (■), and OM (MG/DG/PC/PG=20/30/40/10) liposomes (♦). Peptide and lipid concentration are, respectively, 1 μM and 80 μM. Panel A shows the spectra for F26W-SStp and Panel B shows the spectra for F35W-SStp.

A


F26W-SStp
120
100
80
60
40
20
0
INT
300
320
340
360
380
400
420
wavelength (nm)
OM
PG/PC
PC
buffer

B

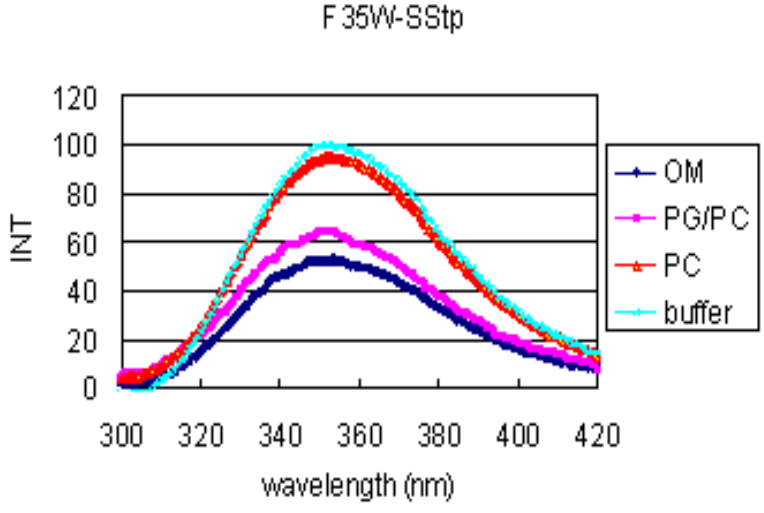

F35W-SStp
120
100
80
60
40
20
0
INT
300
320
340
360
380
400
420
wavelength (nm)
OM
PG/PC
PC
buffer

Table 4-1 Maximum emission wavelength of F26W-SStp and F35W-SStp.

Peptides were tested in the presence of 50 mM Hepes pH 7.6, 0.5 mM DTT and 1‰ TFA, OM, PC/PG, and PC vesicles.

Lipid	Lipid ratio	λmax	
		F26W	F35W
peptide	-	355.5	351.5
+ OM MG/DG/PC/PG	20/30/40/10	350	350.5
+ PC/PG	60/40	350.5	350.5
+ PC	100	353.5	351.5

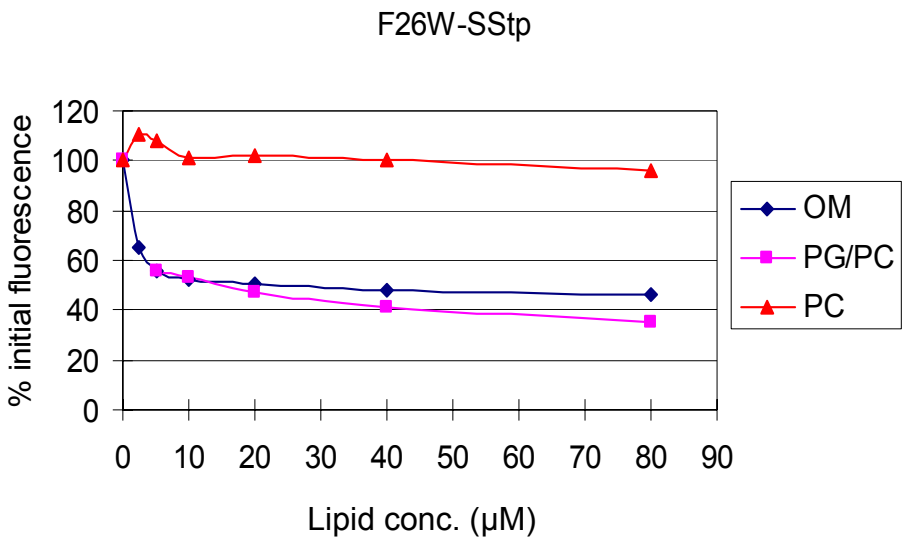
remained relatively constant for both mutants (Figure 4-8), indicating a low affinity of the peptides for this neutral phospholipid. On the contrary, addition of mixed PC/PG (with a molar ratio 60/40) and OM liposomes shifted λmax to lower wavelength and decreased significantly the intensity for F26W-SStp (Figure 4-8 A). This blue shift, indicative of a more hydrophobic environment of the Trp residue at the 26th position, is about 5.5 nm when the protein/lipid concentration ratio is 80 (Table 4-1). In contrast, for F35W-SStp, addition of the same amount of OM and PG/PC lipid vesicles did not shift the λmax much, only 1 nm. This observation suggests that F26 inserts comparatively deeper into the lipid bilayer and the F_{26}TGLK motif is more membrane active. The decrease in fluorescence intensity was still significant for F35W-SStp when the two liposomes were added in, but not as much as that for F26W-SStp.

The corresponding Trp fluorescence intensity titration curves obtained for F26W- and F35W-SStp by plotting the percentage of initial fluorescence as a function of lipid concentration is shown in Figure 4-9. Incubation of both transit peptides with pure PC vesicles had little effect on the Trp fluorescence of both peptides, indicating a weak interaction of the peptides with this lipid. Incorporation of negatively charged PG into PC (PC/PG liposomes) and polar lipids (galactolipids) into PC (OM liposomes) significantly decreased the Trp fluorescence intensity for both peptides. However, the extent of decrease in fluorescence intensity is different for both peptides. The tensity of F26W is decreased more, suggesting that Trp fluorescence is more quenched.

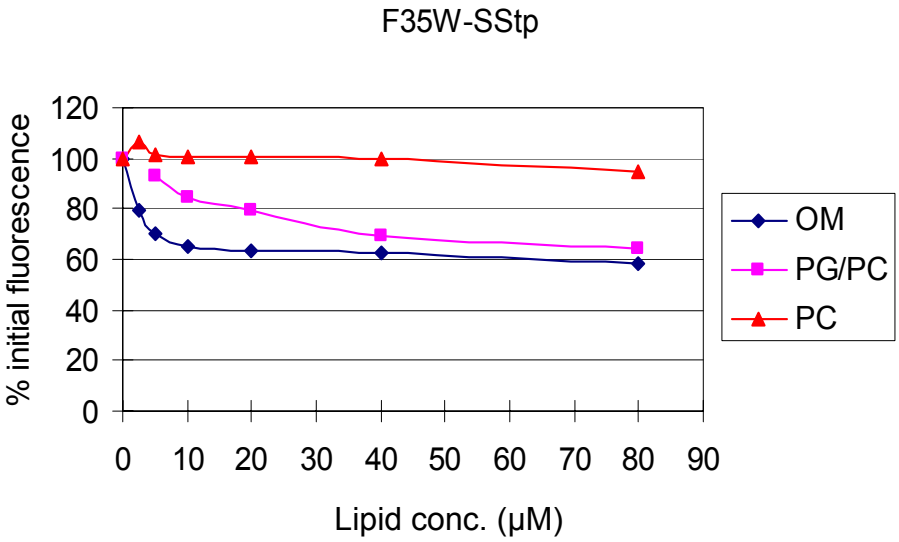
Figure 4-9 Fluorescence titration curves of SStp Trp mutants with different lipid vesicles.

Different liposomes consisting of PC (▲), PC/PG (60/40) (■), OM (MG/DG/PC/PG=20/30/40/10) (♦) were tested. The solid line represents the best fits to the binding curves. 1 μM protein in solution 50 mM Hepes pH 7.6, 0.5 mM DTT and 0.2‰ TFA was titrated in with lipid vesicles.

A


F26W-SStp
% initial fluorescence
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Lipid conc. (μM)
OM
PG/PC
PC

B


F35W-SStp
% initial fluorescence
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Lipid conc. (μM)
OM
PG/PC
PC

4.4 Discussion

4.4.1 Precursors interact with artificial membranes

In the present study we demonstrated that all the substitution mutants were still membrane active. They were able to interact with liposomes, causing release of calcein, originally entrapped in the small unilamellar vesicles mimicking the outer envelope membrane of the chloroplast.

Early calcein dye release assays done in our lab have shown that the C-terminus and N-terminus of SStp have the maximum lipid interacting ability while the central region which contains the two FGLK motifs has little interaction with the lipids (Pinnaduwage & Bruce 1996). This lipid interaction of the transit peptide depended upon the presence of MGDG and anionic lipids (Horniak et al 1993; Pinnaduwage & Bruce 1996). My results agreed well with these experiments. Only a slight decrease of dye release was observed for all the Phe mutants in both motifs compared to the wild-type control, and there was little difference among the Trp, Ser, and Ala mutants on the effect of inducing dye release (Figure 4-1, 4-2, 4-3). The two observations suggest that the Phe residues and the two semi-conserved FGLK motifs are not much involved in the protein-lipid interaction.

Our experiments also confirmed the requirement of specific lipids: galactolipids and PG for the membrane interruption assay, since little/no dye release was detected for neutral lipids PC, even for the most membrane-active wild-type prSSU (panels C & D in Figures 4-1, 4-2, 4-3). Other studies have also indicated that positively charged

peptides don't associate with zwitterionic lipids such as PC (Ben-Tal et al 1997; Keller et al 1996; Hristova et al 1996). All these indicate that electrostatic interactions play a major role in the interaction of transit peptide with the outer envelope membrane of the chloroplast. However, this does not exclude the role of aromatic residues in the interaction with the chloroplast outer membrane lipids. They can interact with them mainly through π-anion interactions, but not hydrophobic interactions.

4.4.2 Partition of peptide into phospholipid bilayer

The leakage of calcein from the interior of the lipid vesicles is due to the disruption of the membrane structure which probably results from the partitioning of transit peptide into the lipids, since mature domain alone causes little release (Figure 4-1, 4-2, 4-3). Partitioning of a peptide into a bilayer involves 3 steps: the adsorption of the peptide to the interface, subsequent conformational changes in the peptide and membrane, and an insertion step (Hunt et al 1997). Upon proximity to lipid vesicles, the peptide is encountering 3 kinds of environments, each with distinct solvent properties: the aqueous phase, the polar headgroup (interfacial region), and the acyl chain region (hydrophobic core). The aqueous phase is a hydrogen-bonding solvent, the acyl chain region is apolar, whereas the interfacial region is a transition phase. It can be both hydrophobic and hydrophilic, containing charges, dipoles, hydrophobic groups (e.g., choline), hydrogen bond acceptors and donors (Voglino et al 1998).

Interfacial partitioning of Lys and Arg were assumed to be relatively favorable because of the possibility of their methylenes interacting hydrophobically with the membrane interface while their charged moieties interact with the aqueous environment (Segrest et al 1992). Aromatic residues are shown to have favorable free energies to partition into membrane interfaces (White & Wimley 1998). In addition, the peptide bond in H-bond is also involved in this process, and this participation greatly reduces its cost of partitioning into non-polar phases. This effect is important for the promotion of secondary structure and is observed when antimicrobial peptides (Maloy & Kari 1995), and signal peptides (Gierasch 1989) partition into a membrane interface. This process was called partitioning-folding coupling (Wimley & White 1996). A typical example of this process is observed using 26-residue melittin (White et al 1998). Melittin is monomeric and shows random coil in aqueous solution, but upon partitioning into bilayer interface, a highly helical conformation is introduced.

According to the analysis above, SStp has a good chance to partition into the interface of lipid vesicles. It is positively charged (3 positive charges in the FGLK motifs) and is rich in hydrophobic residues that are greatly favored for partitioning. It might partition into the lipid vesicle and secondary structure may be introduced, which is thought to be the common, recognizable motif for proteinaceous translocons. The promotion of secondary structure upon association with OM liposomes is observed for SStp by CD experiment (data not shown). Then the participation or the formation of secondary structure of the transit peptide causes the reorientation of the lipid

molecules (Chupin et al 1994), and thus the leakage of calcein dye. This change in lipid organization can directly be involved in protein import (Killian et al 1990) or be required for the activation of the import machinery.

For the effects of mutation of the aromatic Phe residue, Trp mutants at both positions still had the highest membrane activity compared to wild type prSSU, as was the case in the import competition assay. Ala and Ser mutants behaved very similarly and had less membrane activity in all the mutants. Considering that aromatic residues are highly favored in the partitioning process, substitution with Ala and Ser is expected to influence the partitioning process (less membrane activity), so a less amount of dye was released by the F→S and F→A mutants.

Concluding remarks for the calcein release assay:

The dye release data indicates that these mutants show the same behavior trend as in the import competition assay. The membrane activities of all mutants were slightly decreased due to the substitution, with Trp mutants showing the best. Comparing the effect of substitution, the effect on the import competence is much greater than that on the membrane activity. This indicates that in addition to the interaction with membrane lipids, there are some significant factors involved in the import process. The aromatic residues may also interact with protein receptor Toc159/86 or Toc34 during preprotein recognition, possibly via π-π interactions as is discussed in chapter 3.

4.4.3 Trptophan fluorescence

Transit peptides F26W-SStp and F35W-SStp showed comparable fluorescence characteristics in buffer, with the fluorescence maxima centered at 355 nm and 351 nm, respectively. This is indicative for the tryptophan residues located in a hydrophylic environment (Lakowicz 1999). The difference between the two may be explained by the subtle structural environments in which the two amino acids are located. Insertion of a Trp residue into a more hydrophobic environment is usually characterized by a fluorescence blue shift and an increase in the fluorescence quantum yield (Udenfried 1969). However, this was not observed for any of the three kinds of lipid vesicles used in our study: OM liposomes mimicking the outer envelope of the chloroplast; PC/PG liposomes with a molar ratio 60/40; and liposomes made of PC alone. Addition of neutral PC lipid vesicles to both peptides induced no blue shift and the fluorescence quantum yield remained similar. This suggested only a very weak interaction between the peptides and PC vesicles. However, the presence of anionic lipids and OM altered the fluorescence characteristics of the tryptophan variants in other ways. The fluorescence of F26W-SStp was blue-shifted (5 nm) and the quantum yield decreased. Whereas F35W fluorescence showed little blue shift (1 nm), the intensity still decreased significantly with the titration of liposomes. This is in contrast with the behavior of membrane active typical peptides (Jain et al 1985). Three possibilities might account for this fluorescence reduction as listed below.

1) Intermolecular self-quenching (Lakowicz 1999); the transit peptide could aggregate

upon binding to liposomes. Accordingly, in these aggregates, the Trp residues may be in close proximity to one another, resulting in Trp self-quenching or quenching by the positive charges of the peptide, as suggested for antimicrobial nisin (Breukink et al 1998) and Temporin L (Zhao & Kinnunen 2002). No observation of obvious increase in peptide-induced turbidity helped to rule out this possibility.

2) Intramolecular quenching (Cowgill 1970; Steiner & Kirby 1969); quenching can be caused by surrounding amino acids of the tryptophans (Breukink et al 1998). The side chains of nine amino acids, including lysine and arginine can quench Trp fluorescence (Bushueva et al 1974; Bushueva et al 1975; Chen & Barkley 1998). Clark and his colleagues showed that Trp109 in the cellular retinoic acid-binding protein I was fluorescence-silent due to its interaction with the guanidino group of Arg111 (Clark et al 1996). Similar quenching of Trp158 by Lys165 in the extracelluar domain of human tissue factor was reported by Hasselbacher et al. (1995).

SStp is enriched in positively charged residues and these two phenylalanines each have positively charged residues adjacent to them. Lys30 is three amino acids away from Phe26, and for Trp35, it is in close proximity to two positive charges in row: Arg39 and Lys40. This must account for the attenuation of the quantum yields. The greatest possibility is π-cation interactions of the indole ring with these positive charged residues which shield the fluorescence intensity. This is also suggested by the close proximity of Trp and Lys 7 in α-helical temporin L (Dougherty 1996).

3) Quenching by external groups found in the lipids composing the bilayer (Steiner &

Kirby 1969) is the third possibility. Chloroplast membranes contain specific lipids like MGDG/DGDG and PG whose head groups are possibly involved in the interaction with the indole ring. The charged head group of PG was shown to interact directly with the π-orbital of Trp (de Kroon et al 1990; Christiaenes et al 2002). Our experiment showed indirectly that the polar galactol moiety of MGDG is interacting with aromatic residues too. OM and PC/PG liposomes have similar effects on the fluorescence as can be seen from the titration curves, but they have different lipid composition. PC/PG has 3-fold more PG than OM has, but OM has galactolipids which PG lacks. Since DGDG and PC have already been shown not to interact with proteins (van't Hof &de Kruijff 1995; Pinnaduwadge & Bruce 1996; Ben-Tal et al 1997), it must be MGDG that is interacting with aromatic residues, together with PG in the OM liposomes.

From the difference in fluorescence behavior, we can tell that the two tryptophans at position 26 and 35 become located in different environments upon association with the membrane. Based on the small yet significant blue shift, F26W is postulated to be buried only partially in the hydrophobic core region of the membrane. Since little/no blue shift is detected for F35W, it is postulated that it is positioned even shallower in the bilayer, residing primarily in the region of the hydrophilic head groups. However, early NMR work revealed a different phenomenon for Phe34 in the only FGLK domain of trFd. It is found mostly embedded in the hydrophobic interior of micelles. It is also in the most stable structure of trFd (Wienk et al 2000). But this doesn't mean

it is contradictory to our findings. Since there is only one FGLK motif in trFd, it is very possible that it behaves differently to either of the two motifs present in SStp.

Concluding remarks for Trp fluorescence:

Briefly to say, the transit peptide is located only in the interface of the lipid vesicle and does not insert deep into the hydrophobic core. As is revealed by micelle studies, Silene trFd is localized in the micelle interface, but not the deep core region (Wienk et al 2000). This makes sense, considering that if the transit peptides inserted deeply into the hydrophobic core, it would require much more time and energy to transfer the peptide to proteinaceous receptors, which is not necessary if the transit peptides only have weak interaction with the membrane lipids. The import process is a complicated but fast process, after transient contact with membrane lipids, transit peptide/preproteins need to go on quickly for the next stages.

4.4.4 Overall protein-membrane interactions

This part of the study is aimed at getting a better insight into the interaction of transit peptides with lipids, especially the contribution of the two loosely conserved motifs. Membrane interruption assay revealed that the preprotein-membrane interaction is mediated by transit peptide, but the aromatic residues of the two loosely-conserved FGLK motifs are not actively involved. Upon the binding of the transit peptide to lipid vesicles, the two motifs are positioned in different depths of the membrane, suggesting different roles the two motifs might have in the preprotein import process.

Chapter 5 Conclusions and future directions

5.1 Conclusions

By using 13 Phe mutants in our import competition assay, we have shown that the semi-conserved aromatic residues in the transit peptide of tobacco prSSU are important in the process of protein import into the chloroplast *in vitro*, thus indicating a role of the semi-conserved motif FGLK. Mutation studies showed that the two phenylalanines F26 and F35 have somewhat different roles in the interaction with the import apparatus. F35 has more involvement in the interaction. The decrease in the competitive abilities of these mutant preproteins is not due to a decrease in the interaction with membrane lipids, since they only demonstrated a slight reduction in the membrane disruption assay which utilized the artificial small unilamellar vesicles that mimics the outer envelope membrane of the chloroplast. Tryptophan fluorescence studies using exogenously introduced fluorescence probe Trp amino acids in both F26 and F35 separately, transit peptide mutants F26W-SStp and F35W-SStp revealed a slightly different environment of the two amino acids upon association of membrane, indicating a different role they might have in the import process. These results in combination suggest that the FGLK motifs may participate in protein-protein interaction with one or more component of the Toc/Tic apparatus.

5.2 Future directions

Though our experiments have shown that phenylalanine is involved in the import process and it is not much involved in the interaction with membrane lipids, it would be more helpful in understanding the theory of import process if we tested its interaction with the preprotein receptors in the outer envelope membrane, so that we can tell if the FGLK motifs are involved in the early step of the translocation process, like recognition/binding, or the decrease of the import efficiency of the substitution mutants is because the later stage of transport is disrupted. In addition, mutation of the positively charged amino acids in the two loosely conserved motifs would help to understand if the electrostatic interaction is involved in preprotein recognition/binding and which receptor they are interacting with, since work done by Row and Gray have shown that one or more of the binding components of the chloroplast protein import machinery contain functionally important aspartate/glutamate both in solvent-exposed and hydrophobic environments, as well as some other amino acids (Row & Gray 2001).

Most of our understanding of the import process has mostly relied on biochemical or *in vitro* import study, and they have provided considerable information on receptors, kinetics, and mechanistic details of protein targeting. However, sometimes *in vitro* study cannot faithfully reflect what is going on under physiological conditions. Rensink and his colleagues have found that the transgenic Arabidopsis that had the deletion mutants within the transit peptide of prFd transformed behaved differently

compared to isolated chloroplasts. Wild type prFd was able to be imported and processed to mature size both in transgene plants and isolated chloroplasts, while the deletion mutants cannot import into isolated organelles, but some could import in transgenetic plants (Rensink et al 1998).

Green fluorescent protein is a good probe for *in vivo* assays to visually detect the final location of a protein after import into an organelle. If we generate chimeric GFP with our interested prSSUs, or even use different-colored fluorescent proteins, we can differentiate preproteins from each other. It would provide more accurate information in understanding the mechanism, and through comparison of the amount of proteins accumulated in the stroma of wild type prSSU and mutant prSSU, we can more accurately define the functional motifs of the transit peptide.

Structure is the basis for function. Though the structure of trFd has been heavily studied in different environment (Horniak et al 1993; Wienk et al 1999; Wienk et al 2000), little has been done on SStp. If we can get the structure of SStp, it would be helpful to generalize the structure of different transit peptides and help to clear the paradox of SStp-Toc interaction. Also through the comparison of the structure of wild type to mutant SStp in this study, we can tell that if the decrease of import is due to the change in the secondary or tertiary structure, and what positions the two loosely conserved motifs FGLK are located, thus help to understand their function.

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VITA

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