

Testing FGLK Regions in PrSSU

Christopher M Browne

Senior in Biochemistry, Cellular and Molecular Biology

Abstract:

The special nature of the chloroplast requires that it have a way to import proteins through its outer and inner membranes that are transcribed in the nucleus and translated in the cytosol. The Toc and Tic (Translocon of the Outer and Inner membrane of the Chloroplast) complexes perform this role through a relatively unknown mechanism. Proteins destined for the chloroplast contain an N-terminal targeting sequence, the transit peptide, that is both necessary and sufficient for importation. This study examined the importance for importation of two semi-conserved regions, called FGLK regions, on the transit peptide of the small subunit of the Rubisco (Ribulose-1,5-bisphosphate carboxylase-oxygenase) complex (prSSU) in *Nicotiana tabacum*. To do this, full and partial deletion mutants were generated at the two sites. Results showed that the first FGLK region severely hampered translocation into the chloroplast while the second FGLK deletion had lower import than wild type but to a lesser extent. This shows that these regions interact with factors necessary for importation, the loss of this interaction will impede translocation into the chloroplast.

Introduction:

Two thirds of all proteins in eukaryotic cells must transverse a cellular membrane (Schatz and Dobberstein, 1996). Translocation of proteins across the membranes of organelles such as plastids and mitochondria is facilitated by complexes called translocons (Schleiff, 2000; Schleiff and Soll, 2000). A large amount of work has been devoted to determining the mechanism of protein import into the chloroplast (Keegstra and Froehlich, 1999; Bauer et al., 2000; Jarvis and Robinson, 2004; Kessler and Schnell,

2004; Soll and Schleiff, 2004; Hofmann and Theg, 2005). Plastids, which include chloroplasts in green plant tissues, are widely accepted to have evolved from cyanobacteria through the process of endosymbiosis. The modern plastid contains a genome of only around 150 genes (Bruce, 2001). The majority of chloroplast proteins are encoded by the nuclear genome and are translated in the cytosol (Lee et al., 2006). A typical cyanobacterium contains around 3200 genes, for the chloroplast to have the same level of complexity as its ancestor, several thousand gene products must be targeted back to the plastid (Bruce, 2000). Proteins destined for the chloroplast and other organelles need some sort of signal for effective trafficking. This information is usually a small, cleavable, N-terminal signal sequence. These sequences are called signal peptides for the endoplasmic reticulum, presequences for the mitochondria, and transit peptides for plastids (Bruce, 2000). Transit peptides are both necessary and sufficient for targeting to the chloroplast (Keegstra and Cline, 1999; Vothknecht and Soll, 2000; Bruce, 2001). Interestingly, the majority of outer envelope membrane proteins on the chloroplast are targeted without a cleavable transit peptide (Lee et al., 2001, 2004; Tu et al., 2004; Hofmann and Theg, 2005). The first example of precursor transport was demonstrated in 1978 (Chua et al., 1978; Highfield and Ellis, 1978). Despite the sequencing of the *Arabidopsis* genome and discovery of more than 3500 proteins targeted to the chloroplast during the life of a plant, the common mechanism of their translocation is not well understood. One problem is that transit peptides have widely divergent length, composition and organization (Bruce, 2001).

The translocon complexes for the envelope membranes of the chloroplast comprise the Translocon for the Outer and Inner envelope membranes of Chloroplasts,

Toc and Tic. The initial step in translocation is recognition of the preprotein by receptors (Schnell et al., 1997; Hinnah et al., 1997). Though mentioned later, it is not known if the preprotein approaches the receptor from the cytosol, or diffuses there from the surface of the chloroplast or comes bound to other factors like molecular chaperones. Two proteins have been identified to act as the receptors for Toc mediated translocation, Toc34 and Toc159 (Soll et al., 1988; Sveshnikova et al., 2000; Seedorf et al., 1995). After receptor binding, translocation occurs through the membrane channel protein Toc75 (Schnell et al., 1997; Hinnah et al., 1997).

A quintessential preprotein for studying import dynamics is the preprotein of the small subunit (prSSU) of ribulose-1,5-bisphosphate carboxylase-oxygenase (rubisco) (Schleiff et al., 2002). PrSSU was the first protein shown to import post-translationally into chloroplasts (Dobberstein, 1977). It has been shown that prSSU binds directly to both Toc34 and Toc159. The phosphorylation of Toc34 stops its association with prSSU (Sveshnikova et al., 2000). Recognition of the preprotein by Toc34 and Toc159 has been shown to be enhanced by addition of guanosine triphosphate (GTP) (Sveshnikova et al., 2000; Young et al., 1999; Kouranov and Schnell, 1997). In *Arabidopsis thaliana* it has been shown that prSSU may be specifically bound and imported by a developmental isoform of Toc34, atToc33 (Gutensohn et al., 2000). Figure 1 shows the general import

pathway for precursor import (Bruce, 2000).

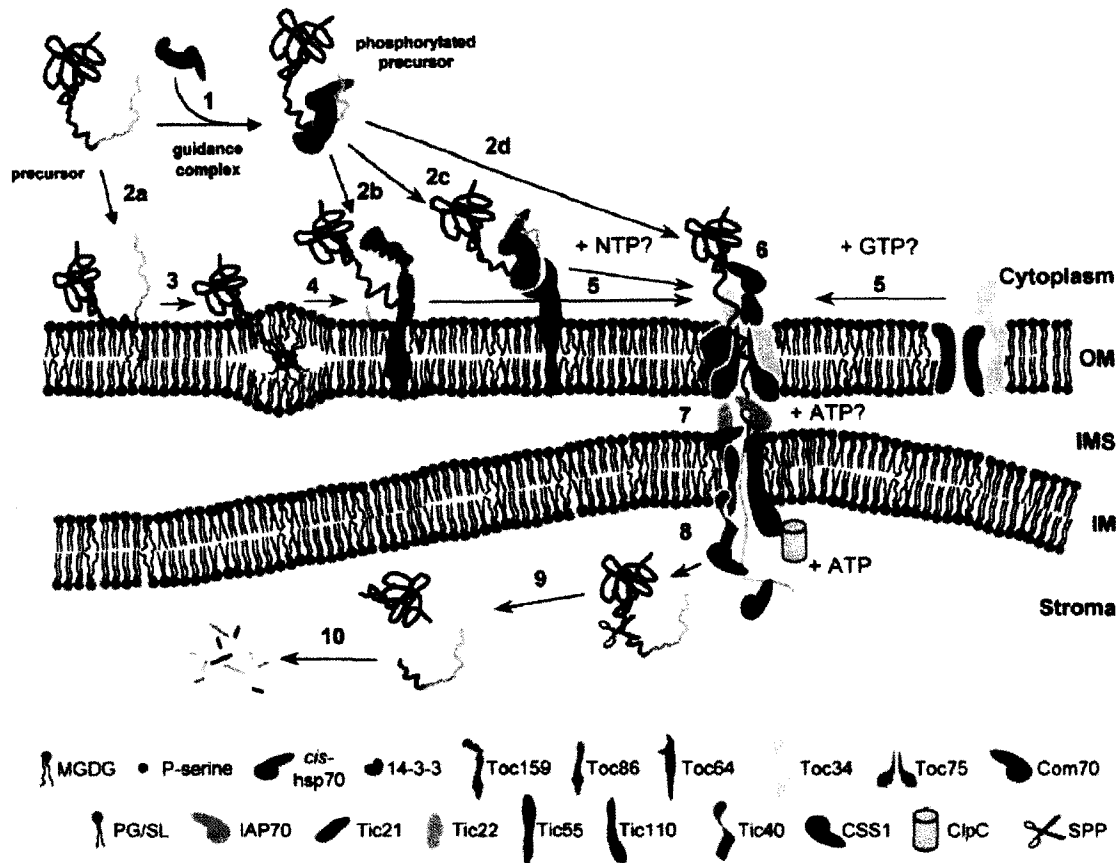


Figure 1

General import pathway for plastid precursor import. Three hypothetical domains of the transit peptide are shown in red, green and yellow. The multiple steps of transit peptide-mediated protein import are shown by the numbers and are described below. (1) Interaction of the transit peptide containing a phosphorylated serine with soluble guidance complex containing a 14-3-3 protein and a *cis*-acting hsp70. (2a) Partitioning of the precursor out of the cytoplasm onto the chloroplast surface via a direct NTP-independent interaction of the transit peptide with chloroplast-specific lipids, such as MGDG, SL and PG. (2b, 2c, 2d) Direct interaction of the precursor Toc components, possibly facilitated by recognition of components in the guidance complex. Interaction may be initially with the full-length Toc159 (2b), Toc64 (2c), or the heteroligomeric Toc translocon (2d). (3) Peptide/lipid interactions resulting in reciprocal changes in both the transit peptide structure (shown as a green helix) and the lipid phase preference of the envelope (shown as an inverted micelle). (4) Recognition and interaction of membrane-associated transit peptide with Toc86/159 receptor. (5, 6) Lateral movement and/or transfer of the transit peptide from the initial association with Toc86/159 and/or Toc64 to assemble with Toc34 and Toc75, resulting in the creation of a Toc translocon, possibly localized at a contact site containing both the inner and outer envelope. This also illustrates (6) the sequential or concurrent GTP-driven insertion of transit peptide into Toc75. (7) Precursor translocation across the outer envelope membrane by a push-pull mechanism using the ATP-dependent molecular motor(s) Com70 and/or IAP70. (8) Precursor translocation across the inner envelope membrane by a push-pull mechanism using the ATP-dependent molecular motor(s) IAP70 and/or CSS1. (9) Transit peptide recognition and cleavage by the stromal processing peptidase. (10) Rapid degradation of the 'free' transit peptide in the stroma by some unknown peptidase(s) (Bruce, 2000).

The importation of proteins post-translationally leads to several problems for a cell. It allows a preprotein the possibility of folding outside its normal environment, a folded preprotein that gains its activity at the wrong time can have dire consequences for a compartmentalized eukaryotic cell. Though the Tat pathway exist in bacteria and the thylakoid membrane of the chloroplast for importing folded proteins (Mori and Cline, 2001), the majority of protein import in plastids is with unfolded proteins (Jarvis and Soll, 2001).

There are few highly conserved blocks of sequence among transit peptides, thus implicating their secondary and tertiary structure in their activity. Only limited information is available concerning the structure of transit peptides (Von Heijne et al., 1989; Bruce, 2000; Zhang and Glaser, 2002). Figure 2 shows that in a aqueous environment transit peptides are largely unstructured, in other solvents though the transit peptide gains some structure.(Bruce, 1998; Wienk et al., 1999; Krimm et al., 1999).

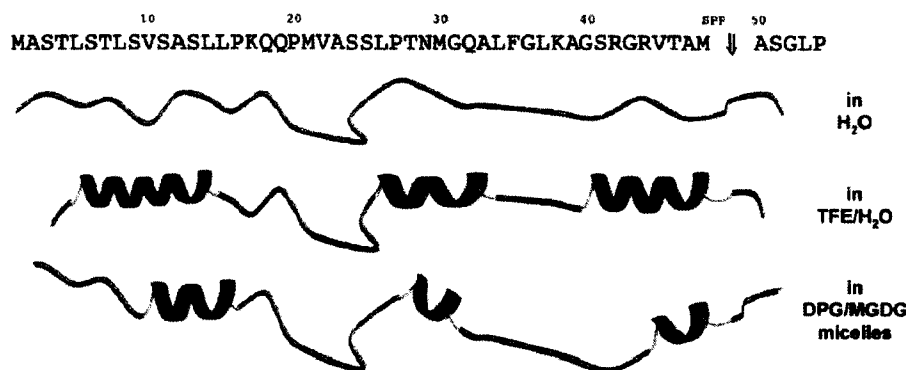


Figure 2

The amino acid sequence of the transit peptide used in the structural studies is displayed in one-letter code at the top. The arrow denotes where the stromal processing peptidase (SPP) cleaves the transit peptide from the mature domain. The top structure represents the lack of structure observed when the transit peptide is in an aqueous solution. Below that is a representation of the helical regions form in the presence of TFE (Wienk et al., 1999). Analysis in mixed micelles of dodecyl phosphocholine, dodecyl phosphoglycol, and monogalactosyldiacylglycerol (DPG/MGDG). In both systems the helical regions are shown as blue/green helixes connected by unstructured regions.

common helical structure in all transit peptides and allow for a small number of receptors, such as Toc34 or Toc159, to translocate many different substrates (Bruce 2000).

It is reported that stromal-targeting transit peptides contain three regions: an uncharged N-terminal domain of about 10 residues beginning with MA- and terminating with a G or P, there is a central domain lacking acidic residues but enriched in S and T. The final C-terminal domain has a number of arginines and could potentially form an amphiphilic beta strand. (Claros et al., 1997).

It has been proposed that the transit peptide is composed of multiple domains with several functions involved in import (Von Heijne et al., 1989; Pilon et al., 1995; Rensink et al., 1989, 2000). In addition to binding to Toc159, Toc75, and Toc34 in vitro (Gutensohn et al., 2000; Hinnah et al., 2001; Jarvis and Soll, 2002; Becker et al., 2004; Kessler and Schnell, 2004; Smith et al., 2004), chloroplast transit peptides also contain domains and motifs for cleavage by the stromal processing peptidase (Richter and Lamppa, 1999, 2002) and DnaK-/HSP70 binding sites (Rial et al., 2000). It is predicted as much as 75% of chloroplast precursor proteins have at least one DnaK binding site (Rial et al., 2000). It has been shown that DnaK/HSP70 binds to its predicted binding site in both prSSU and ferredoxin-NADP⁺ reductase precursor (Ivey and Bruce, 2000; Ivey et al., 2000; Rial et al., 2000). There is also evidence that transit peptides contain a 14-3-3 molecular chaperone binding site (May and Soll, 2000; Bruce, 2001). When prSSU is translated in wheat germ lysate, it is isolated in a complex with a 14-3-3 protein and an Hsp70 protein. This complex imports as much as fourfold faster than prSSU alone (May and Soll, 2000). The binding of the 14-3-3 protein requires that a serine on

the transit peptide be phosphorylated, along with bound HSP70 this may induce the formation of a guidance complex in the cytosol for importation (May and Soll, 2000). Conflicting evidence shows that importation of the precursor is either more efficient in this phosphorylated complex or that importation is not affected by phosphorylation (Nakrieko et al., 2004). In trafficking of proteins in other organelles, soluble targeting factors have been shown to keep precursors in an import-competent conformation and increase fidelity of import (Schatz and Dobberstein, 1996). It also thought that chloroplast precursor proteins interact with lipids at the chloroplast surface prior to initiating importation (Bruce, 1998).

The outer membrane of plastids contain a large proportion of non- bilayer forming lipids. Plastids contain a large amount of monogalactosyldiacylglycerol (MGDG), which prefers to form an inverted hexagonal phase when isolated. Figure 1 (3) has a representation of this inverted hexagonal phase and its association with the transit peptide. This has been shown to be necessary for several steps in translocation (Endo and Schatz, 1988; Retveld et al., 1995). The interaction of the transit peptide and the membrane is enhanced by the presence of certain lipids like MGDG (van't Hof et al., 1991; Pinnaduwege and Bruce, 1996). It has also been shown that the transit peptide-lipid interaction requires anionic lipids such as sulfoquinosyldiacylglycerol (van't Hof et al., 1993). The C-terminal 20 amino acids of the transit peptide have been shown to interact with the chloroplast outer envelope (Pinnaduwege and Bruce, 1996). This interaction is thought to be the initial step that allows the preprotein to diffuse to the translocation complex more easily (van't Hof and de Kruijff, 1995; Chen and Li, 1998).

Several different sequences have been proposed for a common recognition site for transit peptide cleavage by the stromal processing peptidase. These consensus sequences are extremely varied and are not widely conserved across transit peptides. This fact implicated some sort of secondary structure on the transit peptide or mature domain for recognition by the processing peptidase rather than a defined sequence (Bruce, 2001).

A number of different experiments have shown that there is a difference in in vivo and in vitro importation. Mutations in the transit peptide have more negative impact on in vitro import than in vivo. The N-termini of the transit peptide is required for in vivo import, but severe impairment of the transit peptide still leads to in vivo importation that could be sufficient for biological viability (Kindle, 1998; Kindle and Lawrence, 1998; Rensink et al., 1998; Pilon et al., 1995; Lawrence and Kindle, 1997).

The *synechocystis* genome offers some insights into the origin of the Toc complex (Bolter et al., 1998; Reumann et al., 1999; McFadden, 1999). It seems Toc75, Tic22, and Tic20 all have homologues in this cyanobacteria's genome. SynToc75 is the homologue of Toc75 and seems to have been a transporter for virulence factors out of the cyanobacteria. Upon the endosymbiotic incorporation of the cyanobacteria into a eukaryotic cell, SynToc75 became inverted and was thus able to transport proteins into the plastid. Figure 4 shows this switch in functions of SynToc75 and the transfer of the

gene encoding it into the eukaryotic genome (McFadden, 1999; Bruce, 2000).

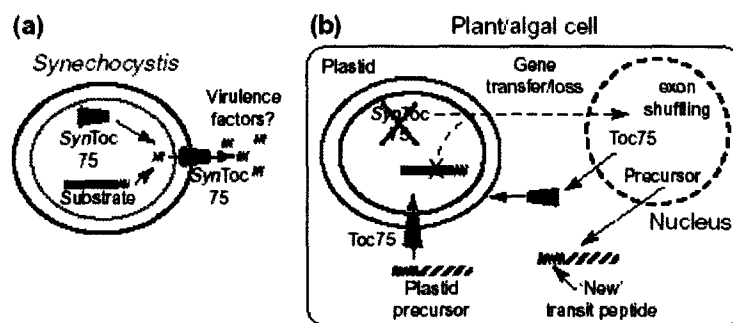


Figure 4

Model demonstrating possible evolutionary origin of Toc75 and modern transit peptides. (a) Possible role of SynToc75 in *Synechocystis* in facilitating the secretion of virulence factors. The substrates for this transporter might include sequences (shown in green) that are recognized by the SynToc75 and might provide the evolutionary origin of at least one domain of the modern chloroplast transit peptide. (b) Movement of genes encoding both SynToc75 and substrate(s) out of the cyanobacteria into the new host genome. The Toc75 protein is now inserted back into the plastid outer membrane in such a way that it now facilitates protein import back into the organelle. Some of the original information recognized by SynToc 75 (shown in green) is rearranged by exon shuffling, eventually yielding a modern transit peptide with the addition of new functional domains (shown in red and blue) positioned at the N-terminus of the 'new' precursor (McFadden, 1999; Bruce, 2000).

The modern transit peptide was probably some sequence already contained in the cyanobacteria's genome. One possibility is it was derived from the sequence that targeted the virulence factors to the SynToc75 complex for export (Bruce, 2001).

The Toc complex is comprised of Toc75, Toc159 and Toc 34; it translocates proteins in a GTP dependent manner (Schleiff et al., 2003). While Toc75 acts as the pore for protein translocation, Toc159 and Toc34 act as receptors for the preprotein. While there is considerable evidence to show that the transit peptide interacts with these receptors, there is also evidence that the mature domain of the precursor may play a role in import as well (Dabney-Smith et al., 1999). Toc159 seems to be the dominant receptor on chloroplast membranes for initial translocation (Kessler et al., 1994; Hirsch et al., 1994). Initially, the Toc159 receptor was identified as Toc86; it was later found that

Toc86 is a common cleavage product of Toc159 (Becker et al., 2004). Toc159 has several isoforms in the Arabidopsis genome: atToc159, atToc132, atToc120 and atToc90 (Hiltbrunner et al., 2001). It is believed that Toc159 may act to import photosynthesis related proteins while Toc132 and Toc120 import proteins in other plastids in the plant not used for photosynthesis (Bauer et al., 2000). The N-terminus of prSSU binds strongly with Toc159 while the C-terminus of the preprotein induces GTP hydrolysis of the receptor when the preprotein is not phosphorylated (Becker et al., 2004). Toc34 is inactivated by phosphorylation, it only binds GTP and preproteins when it is not phosphorylated (Jelic et al., 2002). It is possible that Toc34 and Toc159 act together in the Toc complex to activate the GTPase activity of each other (Powers and Walter, 1995). The initial binding of the preprotein to the receptor is NTP-independent, it seems to be based on a chemical equilibrium (Perry and Keegstra, 1994; Ma et al., 1996).

It has been proposed that there are several regions (FGLK) on chloroplast and mitochondrial targeting sequences that are semi-conserved throughout a number of genomes (Bruce, 2001). Originally labeled as motifs this title may be a bit misleading as these areas of sequence seem too varied across transit peptides to be called motifs. Figure 5 shows that these regions are vastly more prevalent in chloroplasts (ctp) and mitochondria (mtp) than other organelles (McWilliams and Bruce, unpublished). At the top of Figure 5 is the query that was used to generate the graph, the large number of amino acids used in the query that did not fit the FGLK rule shows the relatively minor level of conservation among these regions, though the graph shows they exist none the less.

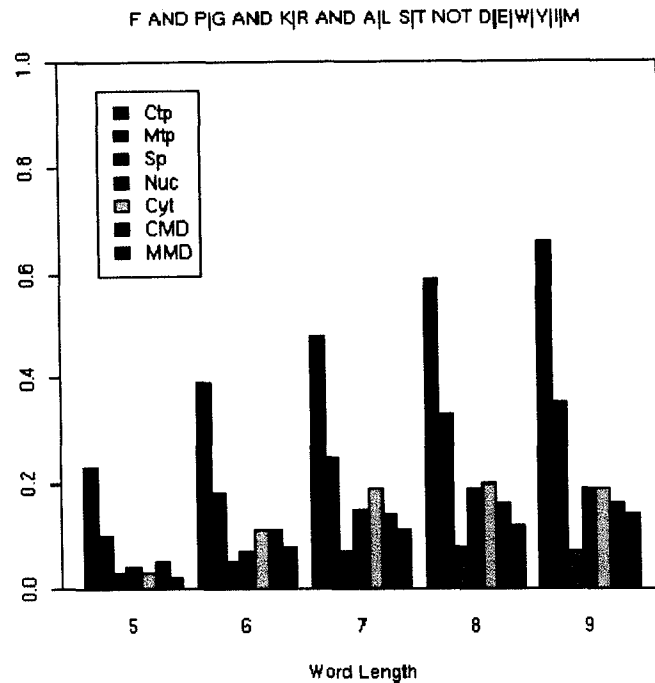


Figure 5

Relative prevalence of FGLK regions in proteins targeted to various organelles. FGLK regions on proteins destined for the chloroplast and to a less extent mitochondria are more common than on proteins destined for other areas such as secretion, the nucleus or the cytosol.

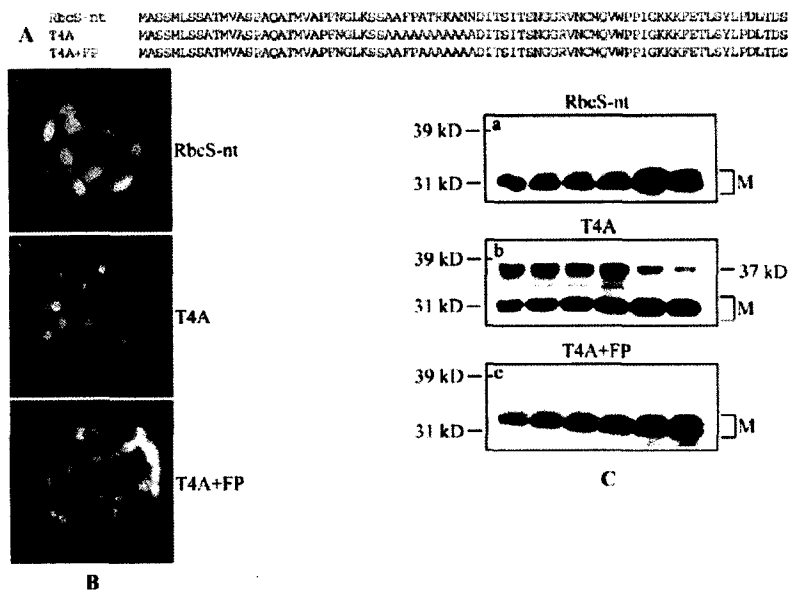


Figure 6

Alanine scanning mutations used to determine important residues for import into *Arabidopsis* chloroplasts. (A) Sequences of wild type prSSU (RbcS-nt) versus a ten amino acid alanine mutation and the same mutation but with an unchanged phenylalanine

and proline. (B) Fluorescence microscopy of *Arabidopsis* protoplasts isolated from leaf tissue. The green is prSSU linked with GFP to visualize the level of import. The red color is the natural fluorescence of chlorophyll. The low levels of green for T4A (b) indicates very little import of the mutant. The high levels of green for T4A+FP (c) indicates the addition of these two amino acids recovers import. (C) Western blots of protoplast import at increasing time points. The 31 kD band indicates successful import with the transit peptide having been cleaved off by SPP, only the mature domain remains. The 37 kD band indicates unsuccessful import, as in T4A (b). Yet again, the FP segment restores import in the mutant (Lee et al., 2006).

A recent study has confirmed that at least one of these regions is important for precursor import (Lee et al., 2006). Figure 6 shows an example from Lee et al. of how a full alanine mutation that stopped import was probed to determine key residues.

The aim of this study was to verify the importance of these semi-conserved regions in the transit peptide of the small subunit of the Rubisco complex. Import competition between wild type prSSU and several deletion mutants of prSSU was used to gauge the necessity of these regions for import into the chloroplast.

Materials and Methods:

Growth of Peas and Chloroplast Isolation

Peas were grown at 20°C with 16 hrs daylight to 8 hrs night for 10 days. Plant tissue was macerated then fully ground using a Polytron grinder at speed 7 in a suitable grinding buffer. Intact chloroplasts were isolated on a Percoll gradient. Quantization of chlorophyll was performed at wavelengths 663 nm and 645 nm in a spectrophotometer, chlorophyll was mixed with 80% acetone to precipitate any starch. Isolated chloroplast solution was brought up to 1 mg/ml of chlorophyll in import buffer (100 mM HEPES, 660 mM Sorbitol, pH 8.0) (IB).

Deletion Mutagenesis of prSSU

The QuikChange Site-Directed Mutagenesis Kit from Stratagene was used to create the deletion mutants. PetII primer containing wild type prSSU from *Nicotiana*

tobaccum was added with nucleotide primers from Sigma or Fisher/Operon and then run through a PCR machine at variable settings. No single set of conditions was able to successfully make all the deletions but multiple tries with slightly varied annealing temperatures were eventually successful for all but one of the planned deletions (Δ FPV). Dpn1 was used afterward to destroy the parental plasmid DNA. PCR products were then transformed into XL1-blue supercompetent cells or nova blue competent cells. There was little difference observed in transformation efficiency between the supercompetent and competent cells. Colonies from the transformation were picked and grown to purify plasmid for sequencing.

Purification of Transit Peptides

Plasmids were transformed into a suitable expression vector, BL21(DE3), and grown in LB to an OD₆₀₀ of around 0.6. They were induced for three hours with IPTG at 1 mM and then pelleted. The pellet was frozen at -80°C for at least two hours. The pellet was resuspended in around 25 ml of buffer A (250 mM Tris-HCl pH 7.6, 25 mM MgCl₂) and lysed by sonication for two minutes at a setting of 6, a glass homogenizer could also have been used at this step. The mixture was spun at 21,000 rpm in a SS-34 rotor for 10 minutes and the supernatant was poured off. The pellet was resuspended in 25 ml buffer A plus 0.1% Triton X-100 and sonicated again at setting 6 for two minutes. This was spun again at 21,000 rpm and the supernatant poured off. This detergent wash was repeated two more times then followed by two similar washes in buffer A alone. The pellet was then resolubilized in 5 ml of 8 M urea solution (8 M urea, 50 mM DTT, 20 mM Tris-HCl pH 8.0). This is spun at 21,000 rpm for 20 minutes, the supernatant is

kept. A Bradford protein assay was then used to determine the concentration of protein purified. The samples were then diluted to 2 mg/ml of protein.

Purification of Radioactive prSSU

BL21(DE3) cells were transformed with wild type prSSU plasmid and grown to OD₆₀₀ of 0.6. After 3 hours of inoculation the cells were pelleted and resuspended in 6 ml of methionine/cysteine deficient media such as 1x EMEM from ICN Biomedicals. The cells are then pelleted again to wash away residual LB media then resuspended in methionine/cysteine deficient media again. The cells were then grown for six hours to deplete the internal stores of methionine and cysteine. The cells were then induced using IPTG at 1 mM concentration. After 5-10 minutes of induction ³⁵S amino acids are added at about 4.2 mCi per 6 ml culture. The cells are labeled for about an hour at 37°C. The cells were then pelleted at 3000 g for 5 minutes and washed twice by resuspension in buffer A and centrifugation at 3000 g. Then protocol for purification then followed that of the non-radioactive protein purification but the wash volumes were around 5 ml each instead of 25 ml.

prSSU Competition Assays

Assays were performed using radioactive prSSU versus the five deletion mutants as competitors plus wild type prSSU as a positive control and the mature domain of the small subunit of Rubisco, mSSU, as a negative control. All competitors were diluted to 1 mg/ml. Five different competitor concentrations were used: 0 nM, 30 nM, 100 nM, 300 nM and 600 nM. Table 1 shows the contents of each reaction.

Competition Assay					
300ul reactions					
in order added (ul):	0 nM	30 nM	100 nM	300 nM	600 nM
1 x import buffer	185.8	185.8	185.8	185.8	185.8
2 x import buffer	19.6	19.6	19.6	19.6	19.6
1 M DTT	3	3	3	3	3
100 mM Mg ²⁺ -ATP	9	9	9	9	9
8 M urea	6.1	4.3	0	4.3	2.4
chloroplasts 1 mg/ml	75	75	75	75	75
³⁵ S-prSSU 1 mg/ml	1.5	1.5	1.5	1.5	1.5
competitors 1 mg/ml	0	1.8	6.1	1.8	3.7
		diluted 1:10	diluted 1:10		

Table 1: Materials and volumes used for competition assays

All materials were added up to the addition of chloroplasts in advance. Chloroplasts were then added and the solution was mixed well. Competitors and ³⁵S labeled prSSU was added simultaneously, import proceeded for exactly 20 minutes then 700 ul of ice cold 1x IB was added to stop the reaction. The samples were layered slowly onto 900 ul 40% Percoll cushions and spun down at 3,000 rpm on a tabletop centrifuge to re-isolate the intact chloroplasts. The top, damaged layer of chloroplasts was aspirated off and 1 ml of 1x IB was mixed to dilute the remaining Percoll. This was then spun down again at 3,000 rpm and the supernatant was aspirated off. The chloroplast pellet was resuspended in 1 ml 1x IB. 50 ul was taken off to run a BCA protein assay so all the samples could be diluted to the same concentrations of protein. The remaining 950 ul was spun at 3,000 rpm and the supernatant was removed. The pellet was resuspended in 25 ul ddH₂O then 25 ul 4x Sample Solubilizing Buffer (SSB) was added. From the results of the BCA assay, the samples were brought to equal concentrations using 4x SSB. The samples

were then boiled for 2 minutes. 20 ul was loaded onto an 18% polyacrylamide gel and run at 8 mAmps per gel overnight, stopping the gels before the sample buffer and green chlorophyll had run off the bottom. The gels were stored in gel destain as the Coomassie dye usually used to visualize protein on SDS-PAGE gels quenches the ³⁵S's radioactivity. Gels were then dried on a gel dryer for around 2 hours and placed on phosphor screens for quantization overnight. The screens were visualized using a Storm840 phosphor screen reader by Molecular Dynamics.

Results:

Six different deletions were planned to test the importance of the two different FGLK motifs on *Nicotiana tabaccum* prSSU; two full deletions and four partial deletions. The sites were -FTGLK- and -FPVSR- on the transit peptide, figure 6 shows the six different sites of deletion.

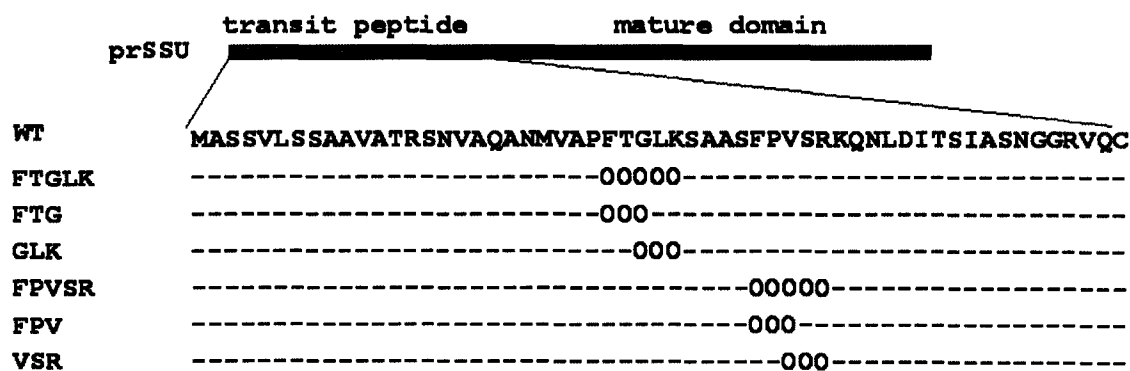


Figure 7

Deletion scheme for prSSU. "0" indicates amino acids deleted while "-" indicates no change from wild type prSSU. Mutagenesis of prSSU ΔFPV was unsuccessful and this mutant was not used in the competition assays.

All six mutations were attempted numerous times but due to low mutagenesis efficiency only five of the six mutations were achieved, Δ FPV was never successful. This meant that the competition assays would use Δ FTGLK, Δ FPVSR, Δ FTG, Δ GLK and Δ VSR as experimental competitors with WT prSSU as a positive control and mSSU as a negative control.

Figure 8 shows the SDS-PAGE gel used to verify that all the purified proteins were at equal concentrations. From the gel it appears that Δ GLK migrated considerably different than the other prSSU mutants.

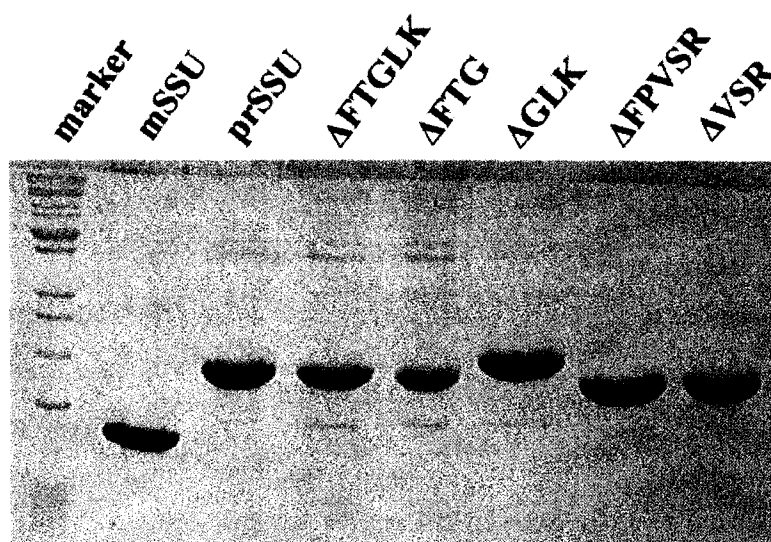


Figure 8

SDS-PAGE gel of purified proteins to be used as competitors all at equal concentrations.

Figure 9 shows the phosphor screen images of the SDS-PAGE gels run from the competition assay products.

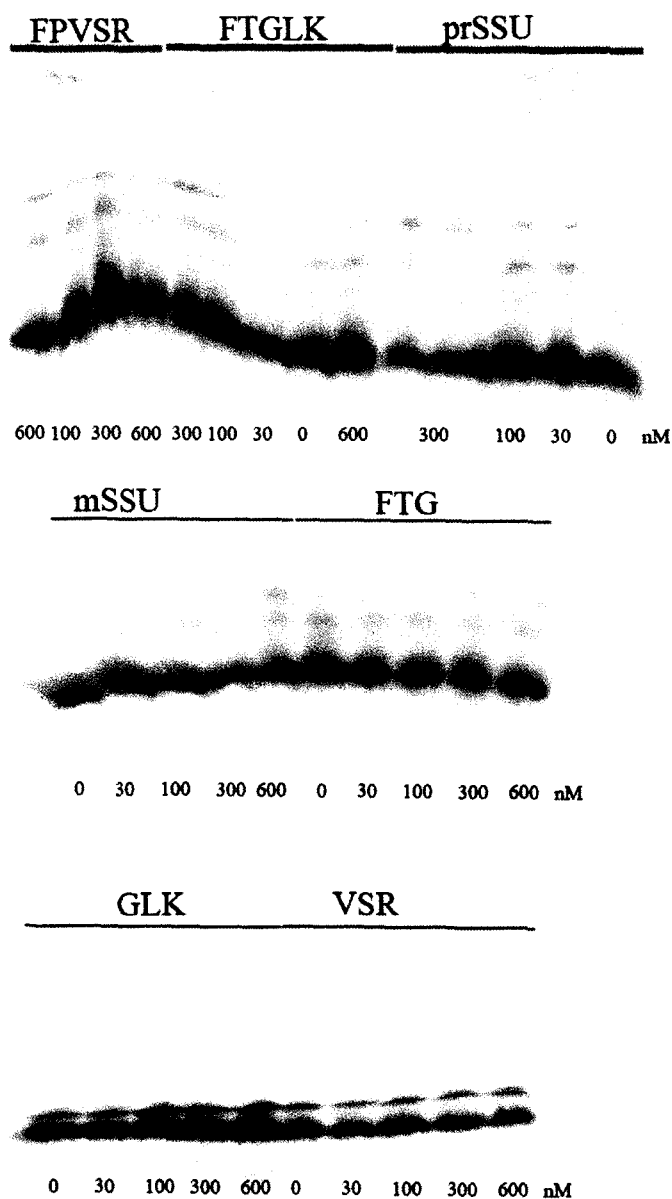


Figure 9

Phospho screen images of dried SDS-PAGE gels. The lanes for the various deletion mutations are underneath each horizontal line. The concentrations of each lane is listed below it.

The levels of imported prSSU as evidenced by these gels shows that the deletion mutants were poor competitors. Without the ability to quantize this data it would be difficult to say that

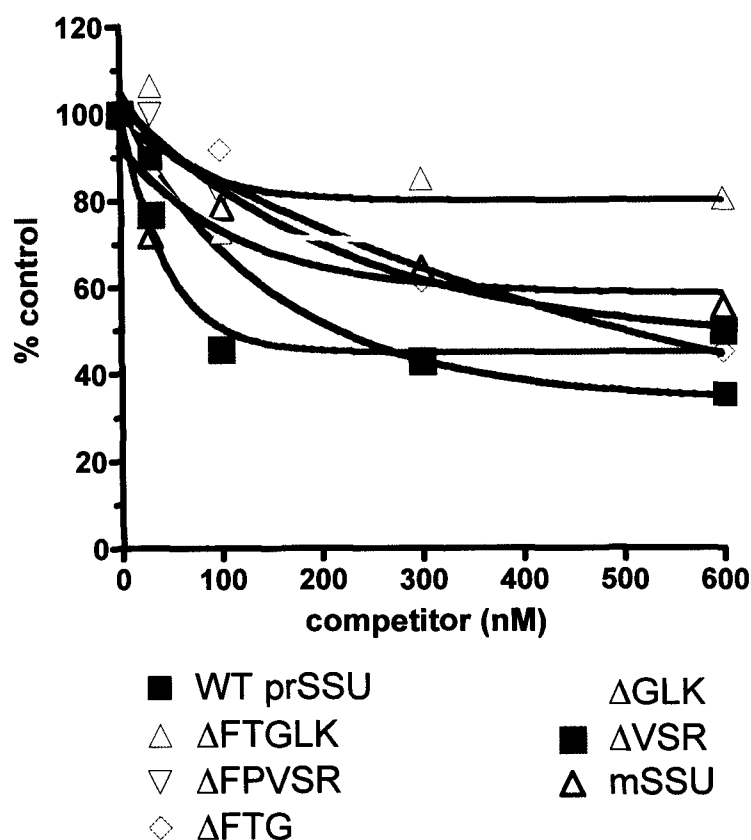


Figure 10
Relative intensity of imported prssu versus wild type prSSU import.

Figure 10 is the graphical quantization of the phosphor screen images. It becomes more obvious that both Δ FTGLK and Δ FPVSR importation was drastically reduced from wild type. The partial deletions likewise had reduced importation but not at the same levels as the full deletions. The full deletion Δ FTGLK show the least evidence of import, the amount of radioactive control prSSU imported stayed constant as the concentration of these competitors rose. The Δ GLK partial deletion showed similar reduced import as the

full deletion. Δ FTG had better import than Δ FTGLK. Though Δ FPVSR had lower import than WT prSSU, its import was still higher than Δ FTGLK. Δ VSR had the highest import ability of all the mutants.

Discussion:

This experiment has shown that the two previously identified FGLK regions on prSSU are important for precursor importation. While both the full deletions of either region lead to reduced importation, the interesting fact is that the Δ GLK partial deletion had the second highest loss of import efficiency. The other partial deletion at the same site, Δ FTG did not have the same impact on precursor import. These two deletions point to the importance of the GLK amino acids for protein import into the chloroplast.

In Lee et al., (2006) it was shown that in *Arabidopsis* the second FGLK region, FPxxRK, was important for import into the chloroplast. Our study verifies that without this region there is lowered import efficiency. Interestingly Lee's findings showed that the first few amino acids of the first FGLK region, FNGLK in *Arabidopsis*, did not have nearly the same impact on import as the second FGLK region. It was only in combination with another alanine substitution further down the transit peptide that import was seriously affected. Our findings contradict this and say that the first FGLK region is more important for importation than the second FGLK region, at least in tobacco. In particular the amino acids GLK seem to have a very significant role in importation. An important point to make is in Lee et al., alanine scanning mutagenesis was used to rapidly change large swathes of the transit peptide to low impact residues. The double mutants that confirmed the importance of a number of sites on the transit peptide had from 19%

and 25% of the total transit peptide mutated to alanines. Even though alanine is a relatively neutral amino acid, the sheer amount of alanine alone could account for the disruption in importation. By totally deleting the sequences in question there is less of a chance that the switched amino acids would have some sort of secondary impact on transit peptide import into the chloroplast.

Obviously, some problems will need to be resolved in the future to further verify these findings. For starters the competition assays were only conducted once for each individual competitor and concentration. Several competitors are missing certain concentrations because of accidents during pipetting and centrifugation. Δ FPVSR in particular is missing two different concentrations, 30 nM and 100 nM, which makes its results suspect. Without multiple trials the conclusions of this experiment are preliminary at best and will need to be further verified. Also, one of the planned partial deletion mutants, Δ FPV, was not successfully mutated and thus data on the second FGLK region on prSSU is incomplete. This problem can be easily corrected and its data can be used in subsequent competition assays.

Future studies should be aimed at repeating and verifying the results already found. The Δ FPV deletion should be mutated and the protein purified for testing. Though it appears from these initial findings that Δ FPV would not have as much impact on importation as the Δ GLK mutation. Double deletion mutants could also be generated to see if this further reduces competitor import. Also, radioactive protein should be made from the deletion mutants to run a time course of import assays. This would not only give a second experiment to further confirm any results, but import assays would be a more direct measure of import efficiency. Competition assays rely on indirectly

measuring the mutant protein by seeing how much wild type protein is also imported in its presence. In Lee et al., *Arabidopsis* transit peptide and chloroplasts are used to test import and shows different results from this study. It would be interesting to create a similar set of deletion mutants in *Arabidopsis* to see if its transit peptide FGLK regions really behave differently from *Nicotiana*'s regions.

An important point to remember is that it is not known what role these FGLK regions have in import. Though they are necessary, the factors they bind to are unknown. For a clearer picture of key players in importation these binding partners must be known. The transit peptide is known to bind with elements of the Toc complex on the surface of the chloroplast as well as HSP70 proteins and 14-3-3 chaperones. The FGLK regions may bind with one of these proteins or maybe something not yet discovered.

Acknowledgements:

I would like to thank Dr. Barry Bruce for his inspiration and critical comments on this project. I would like to thank all members of the Bruce lab both past and present for their expertise and guidance on this project. In particular I would like to thank Sarah Wright for her extensive help in training me over the past three years and for her guidance and helping hand in completing this project. I would like to thank David McWilliams for performing the initial bioinformatics work that lead to this investigation. I would also like to thank Anton Mitsky for help with the chloroplast isolation.

References:

Bauer J., Chen K., Hiltbrunner A., et al. (2000) *Nature*. 403, 203-207.

- Becker T., Jelic M., Vojta A. (2004) *EMBO J.* 23, 520-530.
- Bolter B., et al. (1998a) *FEBS Lett.* 441, 59-62.
- Bruce B. (1998) *Plant Mol. Biol.* 38, 223-246.
- Bruce B. (2000) *Trends Cell Biol.* 10, 440-447.
- Bruce B. (2001) *Biophys. Acta.* 1541, 2-21.
- Buck M. (1998) *Q. Rev. Biophys.* 31, 297-355.
- Chen L. and Li H. (1998) *Plant J.* 16, 33-39.
- Chua N. and Schmidt G. (1978) *Proc. Natl. Acad. Sci. U.S.A.* 75, 6110-6114.
- Claros M., et al. (1997) *Curr. Opin. Struct. Biol.* 7, 394-398.
- Dabney-Smith C., et al. (1999) *J. Biol. Chem.* 274, 32351-32359.
- Dobberstein B., Blobel G., Chua N., et al. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 1082-1085.
- Epand R., Hui M., Argan W., et al. (1986) *J. Biol. Chem.* 261, 10017-10020.
- Endo T. and Schatz G. (1988) *EMBO J.* 7, 1153-1158.
- Gutensohn M., Schulz B., Nicolay P., et al. (2000) *Plant J.* 23, 771-783.
- Highfield P. and Ellis R. (1978) *Nature.* 271, 420-424.
- Hiltbrunner A., Bauer J., Alvarez-Huerta M., et al. (2001a) *Biochem. Cell Biol.* 79, 629-635.
- Hinnah S., Hill C., Wagner K., et al. (1997) *EMBO J.* 16, 7351-7360.
- Hinnah S., Wagner R., Sveshnikova N., et al. (2002) *Biophys. J.* 83, 899-911.
- Hirsch S., Muckel E., Heemeyer F., et al. (1994) *Science.* 266, 1989-1992.
- Hofmann N. and Theg S. (2005) *Trends Plant Sci.* 10, 450-457.
- Ivey R. and Bruce B. (2000) *Cell Stress Chaperones.* 5, 62-71.

- Ivey R., Subramanian C. and Bruce B. (2000) *Plant Physiol.* 122, 1289-1299.
- Jarvis P. and Robinson C. (2004) *Curr. Biol.* 14, R1064-R1077.
- Jarvis P. and Soll J. (2001) *Biophys. Acta.* 1541, 64-79.
- Jarvis P. and Soll J. (2002) *Biophys. Acta.* 1590, 177-189.
- Jelic M., Sveshnikova N., Motzkus M., et al. (2002) *Biol. Chem.* 383, 1875-1883.
- Keegstra K. and Cline K. (1999) *Plant Cell.* 11, 557-570.
- Keegstra K. and Froehlich J. (1999) *Curr. Opin. Plant Biol.* 2, 471-476.
- Kessler F., Blobel G., Patel H., et al. (1994) *Science.* 266, 1035-1039.
- Kessler F. and Schnell D. (2004) *Trends Cell Biol.* 14, 334-338.
- Kindle K. (1998) *Plant Mol. Biol.* 38, 365-377.
- Kindle K. and Lawrence S. (1998) *Plant Physiol.* 116, 1179-1190.
- Kouranov A. and Schnell D. (1997) *J. Cell Biol.* 139, 1677-1685.
- Krimm I., et al. (1999) *Eur. J. biochem.* 265, 171-180.
- Lawrence S. and Kindle K. (1997) *J. Biol. Chem.* 272, 20357-20363.
- Lee Y., Kim D., Kim Y., et al. (2001) *Plant Cell.* 13, 2175-2190.
- Lee D., Lee S., Lee G. (2006) *Plant Physiol.* 140, 466-483.
- Ma Y., Kouranov A., LaSala S., et al. (1996) *J. Cell Biol.* 134, 315-327.
- May T. and Soll J. (2000) *Plant Cell.* 12, 53-64.
- McFadden G. (1999) *Curr. Opin. Plant Biol.* 2, 513-519.
- Mori H., and Cline K. (2001) *BBA Mol. Cell Res.* 1541, 80-90.
- Nakrieko K., Mould R. and Smith A. (2004) *Eur. J. Biochem.* 271, 509-516.
- Perry S. and Keegstra K. (1994) *Plant Cell.* 6, 93-105.
- Pilon M., Rietveld A., Weisbeek P., et al. (1992) *J. Biol. Chem.* 267, 19907-19913.

- Pilon M., Wienk H., Sips W., et al. (1995) *J. Biol. Chem.* 270, 3882-3893.
- Pinnaduwaage P. and Bruce B. (1996) *J. Biol. Chem.* 271, 32907-32915.
- Powers T. and Walter P. (1995) *Science*. 269, 1422-1424.
- Rensink W., Pilon M. and Weisbeek P. (1998) *Plant Physiol.* 118, 691-699.
- Rensink W., Schnell D. and Weisbeek P. (2000) *J. Biol. Chem.* 275, 10265-10271.
- Reumann S., et al. (1999) *Proc. Nat. Acad. Sci. U.S.A.* 96, 784-789.
- Rial D., Arakaki A. and Ceccarelli E. (2000) *Eur. J. Biochem.* 267, 6239-6248.
- Richter S. and Lamppa G. (1999) *J. Cell Biol.* 147, 33-44.
- Richter S. and Lamppa G. (2002) *J. Biol. Chem.* 277, 43888-43894.
- Roise D., Horvath S., Tomich J., et al. (1986) *EMBO J.* 5, 1327-1334.
- Schatz G. and Dobberstein B. (1996) *Science*. 271, 1519-1526.
- Schleiff E. (2000) *J. Bioenerg. Biomembr.* 32, 55-66.
- Schleiff E. and Soll J. (2000) *Planta*. 211, 449-456.
- Schleiff E., Soll J., Sveshnikova N., et al. (2002) *Biochemistry*. 41, 1934-1946.
- Schleiff E., Jelic M. and Soll J. (2003a) *Proc. Natl. Acad. Sci. U.S.A.* 100, 4604-4609.
- Schnell D., Blobel J., Keegstra G., et al. (1997) *Trends Cell Biol.* 7, 303-304.
- Schnell D., Kessler J. and Blobel G. (1994) *Science*. 266, 1007-1012.
- Seedorf M., Waagemann K. and Soll J. (1995) *Plant J.* 7, 401-411.
- Smith M., Rounds C., Wang F., et al. (2004) *J. Cell Biol.* 165, 323-334.
- Soll J., Fischer I. and Keegstra K. (1988) *Planta*. 176, 488-496.
- Soll J. and Schleiff E. (2004) *Nat. Rev. Mol. Cell Biol.* 5, 198-208.
- Sveshnikova N., Soll J. and Schleiff E. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97, 4973-4978.

- Tu S., Chen L., Smith M., et al. (2004) *Plant Cell*. 16, 2078-2088.
- van't Hof R., et al. (1991) *FEBS Lett*. 291, 350-354.
- van't Hof R. and de Kruijff B. (1995) *J. Biol. Chem*. 270, 22368-22373.
- van't Hof R., van Klompenburg W., Pilon M., et al. (1993) *J. Biol. Chem*. 268, 4037-4042.
- von Heijne G. and Nishikawa K. (1991) *FEBS Lett*. 278, 1-3.
- von Heijne G., Steppuhn J. and Herrmann R. (1989) *Eur. J. Biochem*. 180, 535-545.
- Vothknecht U. and Soll J. (2000) *Biol. Chem*. 381, 887-897.
- Wienk H., Czisch M. and de Kruijff B. (1999) *FEBS Lett*. 453, 318-326.
- Young M., Keegstra K. and Froehlich J. (1999) *Plant Physiol*. 121, 237-243.
- Zhang X. and Glaser E. (2002) *Trends Plant Sci*. 7, 14-21.