Protein Import into Chloroplasts

Lea Vojta

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Lea Vojta

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Gutachter:

1. **Prof. Dr. Jürgen Soll**
2. **Prof. Dr. Dario Leister**

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Abbreviations

Aa	Amino acid
AMP-PNP	Adenosine-5'-[β, γ- imido] triphosphate = Adenylyl-imidodiphosphate
ATP	Adenosine-5'-triphosphate
ATP-γ-S	Adenosine-5'-O (3-thiophosphate)
β-MeEtOH	β-mercapto ethanol (2- mercapto ethanol)
BSA	Bovine serum albumin
BCIP	5-bromo-4-chloro-3-indolyphosphate-p-toluid salt
bp	Base pair
cap	m^7-Guanosin (5') ppp (5')Guanosin
C-terminus	Carboxy terminus
DeMa	Decylmaltoside
DGDG	Digalactosyldiacylglycerol
DMSO	Dimethyl sulfoxyde
DSP	Dithiobis-succinimidyl-proprionate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GMP-PNP	Guanosine 5'-[β,γ-imido]triphosphate
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine triphosphate hydrolase
GTP-γ-S	Guanosine 5'-[γ-thio]triphosphate
HEPES	N-2-hydroxyethylpiperasin-N'-2-ethasulfonate
Hsp	Heat shock protein
IEP	Inner envelope protein
IPTG	Isopropylthiogalactoside
KPi	Potassium phosphate buffer
m	Mature form of; mature protein
MGDG	Monogalactosyldiacylglycerol
MGDG Synthase	Monogalactosyldiacylglicerol synthase = UDPgalactose:1,2-diacylglycerol 3-β-D-galactosyltransferase
MOPS	Morpholinopropansulfonate
N-terminus	Amino terminus

NAD	Nicotinamide adenine dinucleotide
NaPi	Sodium phosphate buffer
NBT	4-nitrotetrazoliumchlorid-blue-hydrate
NTP	Ribonucleoside-5'-triphosphate
$O.D._{600}$	Optical density at 600 nm
OE33	33-kDa oxygen evolving complex subunit
OEP	Outer envelope protein
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PEP	Phosphoenol pyruvat
PG	Phosphatidylglycerol
PMSF	Phenylmethane sulfonyl fluoride
PPO	2,5-diphenyloxazole
p	Precursor of
RubisCO	Ribulose-1,5-bisphosphate carboxlase/oxygenase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SQDG	Sulfoqinovosildiacylglycerol
SPP	Stromal processing peptidase
SSU	Small subunit of RubisCO
TCA	Trichloroacetic acid
Tic	Translocon of the inner envelope membrane of chloroplast
Toc	Translocon of the outer envelope membrane of chloroplast
Tricine	N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]-glycin
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol

1 Summary

Import of a hybrid construct consisting of the transit sequence of SSU, the N-proximal part of mature Tic110 and the mature SSU into chloroplasts led to the appearance of a soluble stromal import intermediate and the proposal that Tic110 might use a re-export pathway from the stroma to the inner envelope membrane. For full length Tic110 no soluble intermediate has been observed yet. One of the goals of this work was to investigate the import pathway of Tic110 in more detail. In this research the soluble stromal intermediate of Tic110 was observed, its re-export to the membrane was followed, and finally, the intermediate was isolated and co-immunoprecipitated with the stromal chaperones Hsp93, Hsp70 and to a lesser extent Cpn60. The obtained results indicate that Tic110, as proposed, uses a re-export pathway (conservative sorting) during its import into the chloroplast inner envelope membrane. Tic110 also requires stromal chaperones for achieving its native conformation, prior to the insertion into the inner envelope membrane. The pathway for targeting to the intermembrane space of chloroplasts had not been intensively studied yet. For this reason, the analysis of two intermembrane space localized proteins was conducted: Tic22, a 22 kDa Tic-complex protein component, and MGD1, synthase of MGDG, the most abundant galactolipid in nature. Both proteins are nuclear-encoded and synthesized on cytosolic ribosomes with a cleavable N-terminal chloroplast targeting presequence. Tic22 was found to be associated with the outer face of the inner envelope membrane, as well as with the inner face of the outer envelope membrane, even though at a lower level. MGD1 was proposed to be associated with one of the envelopes by weak electrostatic interactions. Import properties of Tic22 and MGD1 and the localization of MGD1 were investigated in this research. Results presented in this thesis show that import of MGD1 is dependent on, and that of Tic22 is enhanced by, but not dependent on, addition of external ATP. Both preproteins need thermolysin sensitive components on the chloroplast surface for successful import. Chemical crosslinking and immunoprecipitation have demonstrated that Tic22 and MGD1 interact with the components of the Toc translocon of the chloroplast outer envelope during their translocation. Import competition experiments showed that both proteins use the Toc machinery of the general import pathway. Therefore, proteins targeted to the intermembrane space seem to use the same translocation mode across the outer envelope as stromal proteins.

2 Zusammenfassung

Der Import in Chloroplasten eines hybriden Konstrukts, das aus dem Transitpeptid von SSU, dem N-proximal Teil von maturem Tic110 und dem maturen SSU besteht, führte zum erscheinen eines löslichen stromalen Importintermediats und zu der Hypothese, dass Tic110 einen Reexport-Weg vom Stroma in die innere Hüllmembran verwenden könnte. Für das Volllängen-Protein Tic110 war kein lösliches Intermediat beobachtet worden. Eines der Ziele dieser Arbeit war den Importpfad von Tic110 ausführlicher untersuchen. In dieser Arbeit wurde das lösliche stromale Intermediat von Tic110 beobachtet, seine Re-Insertion in die Membran wurde verfolgt, und schließlich wurde das Intermediat isoliert und mit den stromalen Chaperonen Hsp93, Hsp70 und in einem kleineren Ausmaß Cpn60 coimmun-gefällt. Die erhaltenen Ergebnisse zeigen, dass Tic110, einen Reexport-Weg während seines Imports in die innere Hüllmembran von Chloroplasten verwendet. Tic110 benötigt stromale Chaperone, um seine native Konformation vor der Insertion in die innere Hüllmembran zu erreichen. Der Import mechanismus, der die Proteine zum Intermembranraum von Chloroplasten dirigiert, war noch nicht intensiv untersucht worden. Deshalb wurde der Import von zwei im Intermembranraum lokalisierten Proteinen analysiert: Tic22, ein 22 kDa Protein-Bestandteil des Tic-Komplexes, und MGD1, Synthase des MGDG, des häufigsten Galactolipid. Beide Proteine werden im Kern kodiert und an den Ribosomen im Cytosol mit einer spaltbaren N-terminalen Präsequenz synthetisiert. Tic22 bindet an die Außenseite der inneren Hüllmembran, und im geringerem Maße an die Innenseite der äußeren Hüllmembran. MGD1 scheint über elektrostatische Interaktionen mit den Hüllmembranen zu interagieren. Das Importverhalten von Tic22 und MGD1 und die Lokalisierung von MGD1 wurden in dieser Arbeit untersucht. Die hier präsentierten Ergebnisse zeigen, dass der Import von MGD1 von extern zugefügtem ATP abhängig ist im Gegensatz zu Tic22, dessen Importeffizienz in Anwesenheit von ATP zunimmt. Beide Vorstufenproteine benötigen Protease-sensitive Komponenten auf der Chloroplast-Oberfläche für den erfolgreichen Import. Chemische Quervernetzungen und Immunfällungen haben gezeigt, dass Tic22 und MGD1 während ihrer Translokation mit den Bestandteilen des Toc Komplexes interagieren. Importkompetitions-Experimente zeigten, dass beide Proteine die Toc Maschinerie des allgemeinen Importwegs verwenden. Daraus folgt, dass diese Proteine des Intermembranraums denselben Weg über die äußere Hüllmembran nutzen wie stromale Proteine.

3 Introduction

Chloroplast originated from an endosymbiotic event, in which an ancestral photosynthetic cyanobacterium was taken up by a heterotrophic host cell that already contained mitochondria (Cavalier-Smith, 2000). Endosymbiotic evolution has resulted in the transfer of genes encoding the vast majority of the protein components of plastids to the nuclear genome (Martin and Herrmann, 1998). In response to this displacement of genetic material, plastids have evolved a system to post-translationally import nuclear encoded preproteins from their site of synthesis on cytoplasmic ribosomes (Keegstra and Cline, 1999, Martin et al., 2002, Jarvis, 2004). The plastid genome encodes 80-200 plastid-localized proteins that are translated on 70S ribosomes and functionally assembled within the plastid (Jarvis and Robinson, 2004). The protein import machinery of chloroplasts has no known functional equivalent in cyanobacteria (Heins et al., 1998, Heins and Soll, 1998). Homologues of import translocon components Toc75, Tic55, Tic22 and Tic20 have been found encoded in the genome of *Synechocystis PCC6803* (Heins and Soll, 1998, Reumann and Keegstra, 1999), but Toc159, Toc34 and Tic110 show no clear prokaryotic origin and might have been added to the translocon during the conversion of an endosymbiont to an organelle. Plant cells regulate the import apparatus in concert with the protein demands of the developing plastids (Mullet, 1998).

3.1 Protein import into chloroplasts

Most of the nuclear-encoded chloroplast proteins are synthesized with cleavable N-terminal presequences that are necessary and sufficient for targeting to the chloroplast and for translocation across the chloroplast envelope (Keegstra et al., 1989, De Boer and Weisbeek, 1991, Cline and Henry 1996). The targeting sequences are sequentially decoded resulting in the localization of the polypeptide to the appropriate organellar subcompartment: outer and inner envelope membrane, intermembrane space, stroma, thylakoid membranes or thylakoid lumen (Keegstra et al., 1995, Cline and Henry, 1996). Targeting sequences range in size from about 30 to 120 amino acids and are enriched in hydroxylated residues and deficient in acidic residues (Figure 1). N-proximal 10-15 residues are devoid of Gly, Pro and charged residues, a variable middle region of the transit peptide is rich in Ser, Thr, Lys and Arg, lacking acidic residues; and a carboxy-proximal region is loosely conserved (Ile/Val-x-Ala/Cys-Ala) and is

the site where proteolitic processing occurs (Von Heijne et al., 1989, De Boer and Weisbeek, 1991). Although there is no apparent similarity in primary sequence among the transit sequences of different precursor proteins, on the basis of import competition studies and transit sequence swapping experiments it was believed that all preproteins use the same mechanism for envelope translocation (Gray and Row, 1995). However, the existence of distinct import pathways has recently been proposed (Kouranov et al., 1999, Nada and Soll, 2004). After translation on cytosolic ribosomes, a cytosolic protein kinase recognizes transit sequences of chloroplast precursor proteins, and is present only in plant extracts, e.g. wheat germ and pea. The protein kinase phosphorylates, in an ATP-dependent manner, one specific Ser or Thr residue within the stroma-targeting domain of the transit sequence (Waegemann and Soll, 1996). Phosphorylation might act as a kinetic signal for targeting, but the translocation of phosphorylated precursor protein is inhibited (Becker et al., 2005). Dephosphorylation is catalyzed by a protein phosphatase, which is probably localized in the outer envelope, and which is required to allow complete import of the precursor into the stroma.

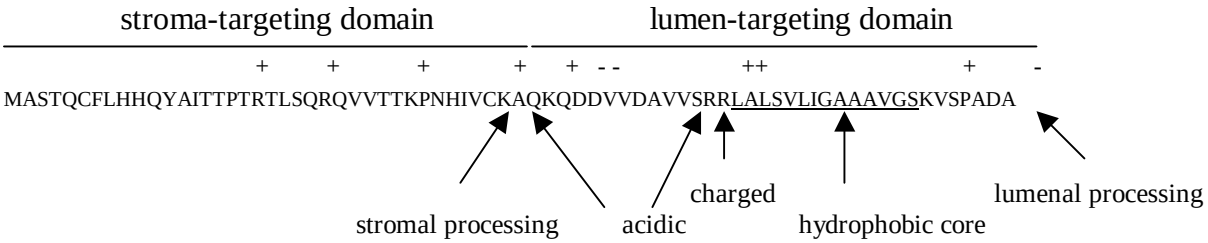


Figure 1. Stroma-targeting domain directs import into chloroplast stroma and lumen-targeting domain to the thylakoid lumen. Stroma-targeting domain is removed by stromal processing peptidase and lumen-targeting domain by a second processing protease (from Cline and Henry, 1996).

Import of precursor proteins into chloroplasts requires protease-sensitive components on the outer envelope membrane (Cline et al., 1985, Friedman and Keegstra, 1989). The lipids found within plastid membranes are also emerging as important players in the targeting, insertion and assembly process of proteins in plastid membranes (Van't Hof et al., 1993, Bruce, 1998, Dörmann and Benning, 2002). The outer chloroplast envelope is strongly negatively charged and enables formation of ionic interactions between preproteins and lipids on the chloroplast surface (Fulgosi and Soll, 2001). Both inner and outer chloroplast envelope membranes are composed of unusual lipids, including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfolipid sulfoqinovosildiacylglycerol (SQDG) and negatively charged phosphatidylglycerol (PG) (Joyard et al., 1991, Pinnaduwage

and Bruce, 1996). The lipid/protein ratio of the outer membrane is very high (around 3) (Block et al., 1983) and has a direct influence on protein binding and import across the chloroplast envelopes.

On their way to the inside of the organelle, proteins have to transverse the outer envelope membrane, the intermembrane space and the inner envelope membrane of chloroplasts. The Toc (translocon of the outer envelope membrane) and the Tic (translocon of the inner envelope membrane) translocation complexes are composed of different protein subunits. They are essential for most of the proteins destined for chloroplasts and their interplay enables successful translocation and targeting to the final destination. For the protein translocation inside the chloroplast stroma, formation of contact sites between outer and inner chloroplast membrane, between Toc and Tic complexes, is needed (Pain et al., 1988, Schnell et al., 1990, Alefsen et al., 1994, Kouranov and Schnell, 1996).

To accomplish successful targeting and import into the organelle, freshly synthesized proteins have to remain in the unfolded conformation. Plant 14-3-3 proteins interact specifically with chloroplast precursor proteins, recognizing their phosphorylated transit sequences. The cytosolic protein 14-3-3 is a molecular chaperone that forms a dimer, where each monomer is able to bind one target precursor protein. It is a component of a 200 kDa hetero-oligomeric complex, in which a 14-3-3 dimer cooperates with Hsp70 and perhaps with additional, yet unidentified components (May and Soll, 2000). The formation of the precursor guidance-complex keeps the preprotein in a highly import-competent state. Dissociation of the precursor complex requires ATP. For around 75% of all plastid precursor proteins at least one site for binding to Hsp70 was predicted (Rial et al., 2000). Chaperones from the Hsp70 family play the important role during protein import into chloroplasts. The chloroplastic outer membrane protein 70 (Com70) is exposed on the cytoplasmic side of the membrane (Ko et al., 1992, Wu et al., 1994). The other, Hsp70 import-associated protein, faces the intermembrane space between the outer and inner envelope membranes (Marshall et al., 1990, Waegemann and Soll, 1991, Schnell et al., 1994).

Multiple pathways exist for targeting and insertion of proteins into the envelope. The first subgroup of proteins destined for the outer chloroplast envelope has internal non-cleavable targeting signals and appears to insert directly into the outer envelope membrane (Salomon et al., 1990, Li et al., 1991, Soll et al., 1992). In this group belong single α-helical transmembrane proteins Oep7, Omp24, Oep14 and Toc34 (Salomon et al., 1990, Fischer et al., 1994, Li et al., 1991, Seedorf et al., 1995) and pore-forming proteins Oep16, Oep21 and Oep24 (Pohlmeyer et al., 1997, Bölter et al., 1999, Pohlmeyer et al., 1998). Those proteins do

no require protease-sensitive receptors on the organellar surface or ATP for the integration into the membrane (Waegemann and Soll, 1995). Toc34, for example, neither contains a cleavable presequence, nor uses the general import pathway. It inserts direcly by its C-terminus into the lipid bilayer, stimulated by GTP, and independent of the presence of charged lipids (May and Soll, 1998, Qbadou et al., 2003).

The second subgroup of OE destined proteins has a cleavable presequence and is represented by Toc75 (Tranel et al., 1995) that needs protease-senstive receptors on the chloroplast surface and ATP hydrolysis for the insertion into the outer envelope membrane. The precursor of Toc75 has a bipartite targeting sequence which N-proxymal part directs the protein to the chloroplast stroma, where it is processed by the stromal processing peptidase to the intermediate-sized form (Tranel et al., 1995, Tranel and Keegstra, 1996, Inoue et al., 2001). The second part of the targeting sequence contains a unique polyglycine stretch that appears to function as a stop transfer domain and is cleaved by plastidic type I signal peptidase (Inoue et al., 2005), creating a mature Toc75 that is subsequently inserted to the outer envelope membrane. For other outer envelope proteins, like Toc159 and Toc64, the topology is not determined so far (Becker et al., 2005).

Most inner envelope proteins are synthesized with cleavable transit peptides and use the general import pathway (Toc and Tic complex, Figure 2) for their translocation. It was suggested that some preproteins contain a hydrophobic stop-transfer signal in their sequence and are released from the translocon on the level of the inner envelope. Others are first targeted to the stroma by their stroma-targeting presequence, and their processed mature form is subsequently re-exported into the inner envelope membrane, by so-called conservative sorting (Lübeck at al., 1997).

Different classes of preproteins seem to exist, which interact preferentially either with Hsp70/14-3-3 or with Hsp90 chaperones. Toc34 recognizes directly preproteins brought by the guidance complex from the cytosol to the chloroplast surface, and is able to recognize and interact with all presequences. Recently it has been shown that some precursor proteins associate with the stromal chaperone Hsp90. In this case, Toc64 acts as an initial docking site for Hsp90-associated precursor proteins, by interacting with Hsp90, and the subsequent transfer of the preprotein from the preprotein-Hsp90-Toc64 complex to Toc34 occurs (Qbadou et al., 2006). Toc34 in its GTP-bound state acts as the initial receptor by binding with high affinity to the transit peptide (Svesnikova et al., 2000, Schleiff et al., 2002, Becker et al., 2004b). Toc34 is converted to its GDP-bound state by preprotein-stimulated GTP hydrolysis (Jelic et al., 2002), resulting in a transfer of the preprotein to Toc159-GTP.

Following dephosphorylation, the preprotein is driven across the outer membrane through the Toc75 channel via a GTP-dependent Toc159 motor in a sewing-machine-like mechanism (Schleiff et al., 2003a). The transfer of the preprotein from Toc34 to Toc159 might be facilitated by the formation of a heterodimer (Smith et al., 2002). Toc159 is the major phosphoprotein in the outer envelope and essential for chloroplast biogenesis (Bauer et al., 2000). It possesses high homology to Toc34 in its GTPase domain (Kessler et al., 1994, Seedorf at al., 1995). The receptors Toc159 and Toc34 are reset to their GTP-bound state and are ready for further recognition and translocation cycles (Kessler and Schnell, 2006). The preprotein is further transfered to the Toc75 translocation channel. It was proposed that Toc75 has a cytosolic preprotein-binding site and itself is able to differentiate between transit peptides on the base of conformational and electrostatic interactions (Ma et al., 1996, Hinnah et al., 1997, Hinnah et al., 2002). The channel opening and pore diameter are proposed to be controlled by regulatory subunit/s. Toc75 and Toc159 form the minimal translocon unit *in vitro* that is able to specifically recognize and translocate chloroplast preproteins across a membrane (Schleiff et al., 2003a). Toc34 represents the initial receptor for incoming preproteins and together with Toc159 and Toc75 forms a Toc core complex (Schleiff et al., 2003b).

Toc64, Toc12, intermembrane space Hsp70 and Tic22 associate together to form intermembrane space portion of the translocation complex (Becker et al., 2004a). The J-domain of Toc12 is proposed to recruit the Hsp70 of the outer envelope membrane to the intermembrane space translocon and facilitate its interaction with the preprotein (Becker et al., 2004a).

Seven protein subunits of the inner-envelope translocon are known: Tic110, Tic62, Tic55, Tic40, Tic32, Tic22 and Tic20. Assembly of functional Tic complexes might be dynamic and occurs in response to preprotein translocation (Kouranov et al., 1998). Tic110 was the first identified component of the Tic translocon and represents a major component of active Tic complexes (Kessler and Blobel, 1996, Lübeck et al., 1996). It is an integral inner envelope membrane protein, and structural predictions suggest that it consists of two transmembrane helices at its extreme amino terminus and a 97.5-kDa carboxyl-terminal region that is largely hydrophilic. Tic110 from *A. thaliana*, atTic110, was shown to be essential for the assembly and function of the protein import machinery of chloroplasts (Inaba et al., 2005). Studies of Tic110 topology and molecular interactions have led to different models for its role in protein import. In the first model, the carboxyl-terminal region of pea Tic110, psTic110, was predicted to extend into the intermembrane space between the outer

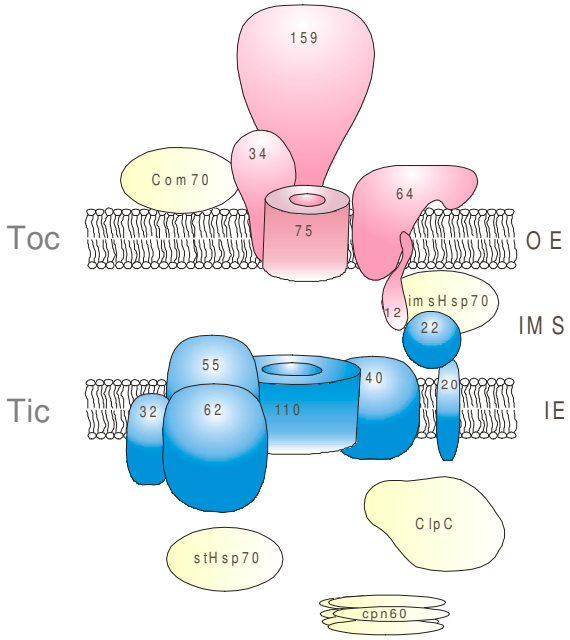


Figure 2. Components of the translocons of the outer (Toc) and the inner (Tic) envelope membrane of chloroplasts. For detailed description see text.

and inner envelope membranes and thereby mediate the interactions between the Toc and Tic complexes during the translocation reaction (Lübeck et al., 1996, Lübeck et al., 1997, May and Soll, 1998). Topology studies performed by Jackson et al. (1998) indicated that psTic110 exists in the opposite orientation with the majority of the protein extending into the stroma. In this model, the large hydrophilic domain of Tic110 was proposed to serve as a docking site for soluble stromal chaperones that assist in the translocation and folding of imported proteins (Kessler and Blobel, 1996, Jackson et al., 1998, Inaba et al., 2003, Chou et al., 2003, Kikuchi et al., 2006). Experiments conducted in our laboratory showed that Tic110 was accessible to proteases from the intermembrane space (Lübeck et al., 1996). Recently, Heins et al. (2002) proposed that Tic110 functions as the protein-conducting channel of the Tic translocon. Tic62 shows strong homologies to NAD(H) dehydrogenases in eukaryotes and to Ycf39-like proteins present in cyanobacteria and non-green algae and is proposed to regulate protein import into chloroplasts by sensing and reacting to the redox state of the organelle (Küchler et al., 2002). Tic55 possesses a Rieske-type iron-sulphur cluster with a mononuclear iron-binding site (Caliebe et al., 1997) and therefore might act as a regulatory subunit that uses the iron-sulfur cluster as a redox sensor to influence the import competence of the chloroplast (Soll and Tien, 1998). It has been proposed that Tic40 plays an accessory role as a co-chaperone in the stromal chaperone complex that facilitates protein translocation across the inner membrane (Chou et al., 2003, Kovacheva et al., 2005). Tic32 faces the stromal compartment of chloroplasts and is essential for chloroplast viability. It shows homology to

short-chain dehydrogenases, and its activity is regulated in a Ca^{2+}/calmodulin dependent manner (Hörmann et al., 2004, Chigri et al., 2006), suggesting a dual role of Tic32 in import: one as a regulatory component that could determine translocation rates across the inner envelope membrane (Chigri et al., 2006) and another as an important subunit in the assembly of the entire complex (Hörmann et al., 2004). For Tic22 it was proposed to serve as a functional link between the translocon complexes in the outer and inner envelopes of chloroplasts (Kouranov and Schnell 1997, Kouranov et al., 1998). Tic20 was shown to participate in the protein import across the inner envelope membrane (Chen et al., 2002) and its role as a part of protein-conducting channel at the inner envelope membrane associated to Tic22 and Tic110 has been proposed (Kouranov et al., 1998, Chen et al., 2000).

In the stroma imported proteins associate with the stromal chaperone Hsp70 and some with the chaperonin Cpn60 (Lubben et al., 1989, Marshall and Keegstra, 1992, Tsugeki and Nishimura, 1993, Madueño et al., 1993). Pea chloroplasts have at least two stromal Hsp70 isoforms: S78 and CSS1 (Kessler and Blobel, 1996). Cpn60 is a member of the Hsp60 family of chaperones and a homologue of the bacterial chaperone GroEL. It could be co-immunoprecipitated with Tic110 (Kessler and Blobel, 1996) only during contact site formation, suggesting its indirect role in chloroplast protein import. Hsp93 was found to be a component of import complexes regardless of whether precursor proteins were present (Moore and Keegstra, 1993, Akita et al., 1997, Nielsen et al., 1997, Kouranov et al., 1998). Hsp93 and Hsp70 might cooperate in pulling the precursors into chloroplasts in a mechanism analogous to that in mitochondria, while Cpn60 assists the newly imported protein in folding into its native conformation (Jackson-Constan et al., 2001).

In vitro protein import into chloroplasts was first demonstrated by Chua and Schmidt and Highfield and Ellis in 1978, using the precursor of the small subunit of RubisCO. The import process can generally be divided into several steps: binding of the precursor to the receptor proteins on the chloroplast surface, translocation across the two envelope membranes and processing of the precursor by the stroma-localized processing peptidase.

Binding of the precursors to the outer envelope membrane constitutes the first step and produces the first stable intermediate, so-called early import intermediate (Theg and Scott 1993, Schnell and Blobel, 1993). Binding requires less than 50 µM NTPs (both ATP and GTP, Soll and Schleiff, 2004) in the cytoplasm or intermembrane space (Olsen et al., 1989, Olsen and Keegstra, 1992, Kessler and al., 1994) and the presence of outer envelope proteins (Olsen and Keegstra, 1992, Theg and Scott, 1993). GTP hydrolysis is necessary for the formation of early-import intermediates, but not for precursor translocation (Young et al.,

1999). Early intermediates are irreversibly bound (Theg and Scott, 1993) and are frequently partially protected from protease treatment of chloroplasts (Friedman and Keegstra, 1989, Waegemann and Soll, 1991). For precursor transport across the outer envelope membrane ATP in the intermembrane space is required (Scott and Theg, 1996) and import through the inner envelope membrane into the stroma progresses if the ATP concentration is raised to around 1 mM (Pain and Blobel, 1987, Theg et al., 1989, Theg and Scott, 1993, Schnell and Blobel, 1993). This ATP is probably needed for the action of molecular chaperones in the stroma, which provide the driving force to complete import into the organelle (Kessler and Blobel, 1996, Nielsen et al., 1997). Upon entering the stromal compartment, the transit sequence is removed by SPP (Robinson and Ellis, 1984, Abad et al., 1989, VanderVere et al., 1995, Richter and Lamppa, 1999).

3.2 Aim of this work

3.2.1 Inner envelope membrane protein Tic110

Tic110 is synthesized with an N-terminal extension of 37 amino acids which functions as classical transit peptide and engages the general import pathway (Lübeck et al., 1996, Lübeck et al., 1997, Jackson et al., 1998). Targeting to the inner envelope membrane appears to require signals within the transmembrane regions of the protein. Studies using chimeric proteins suggest that Tic110 might use a stromal intermediate during its targeting to the inner membrane (Lübeck et al., 1997). A hybrid protein consisting of the transit sequence of SSU, the N-proxymal part of mature Tic110 and the mature SSU (pSSU-Tic110N-mSSU) is completely imported into the chloroplast stroma. N-terminally processed soluble Tic110N-mSSU sorting intermediate then enters a re-export pathway (Lübeck et al., 1997). Furthermore, dominant negative mutants of Tic110 that disrupt Tic complex formation result in the accumulation of normal Tic110 in the stroma (Inaba et al., 2005). These data suggest that at least a subset of inner membrane proteins is re-inserted into the membrane from the stroma after import. Aim of this work was to investigate import properties of Tic110.

3.2.2 Intermembrane space protein Tic22

The pathway of targeting to the intermembrane space of chloroplasts has not been intensively studied yet, due to the lack of a known marker for this subcompartment. Tic22 is nuclear-endoded and synthesized as a preprotein with a 50-amino acids long N-terminal presequence.

Tic22 was identified as a candidate for a component of the general protein import machinery by its ability to covalently crosslink to nuclear-encoded preproteins trapped at an intermediate stage in import across the envelope (Kouranov and Schnell, 1997). Tic22 is a 22-kD protein that is peripherally associated with the outer face of the inner envelope membrane and to a lesser extent to the inner face of the outer envelope membrane (Kouranov et al., 1998, Kouranov et al., 1999).

Kouranov et al. (1999) investigated the import pathway of Tic22 into isolated chloroplasts to define the requirements for targeting of proteins to the intermembrane space. The analysis of deletion mutants and chimeric proteins indicated that the presequence of Tic22 was necessary and sufficient for targeting to the intermembrane space. Import of pTic22 was found to be stimulated by ATP and required the presence of protease-sensitive components on the chloroplast surface. The competition experiments using excess of pSSU indicated that its targeting to the intermembrane space does not involve the general import pathway utilized by stromal preproteins. Kouranov et al. (1999) concluded that the presequence of pTic22 does not function as a stromal transit sequence and that pTic22 is targeted to the intermembrane space of chloroplasts by a novel import pathway that is distinct from known pathways that target proteins to other chloroplast subcompartments. Import properties of Tic22 have been further studied here, aiming to the better characterization of its pathway to the intermembrane space.

3.2.3 Intermembrane space protein MGD1

Galactolipids represent more than 80% of membrane lipids in higher plants, eukaryotic algae and cyanobacteria. 50% of galactolipid content represents MGDG, a monolayer forming, major structural lipid of chloroplasts and non-green plastids and the most abundant membrane lipid in nature (Gounaris and Barber, 1983, Ohta et al., 2000). It is found in plastid envelopes, as well as in thylakoid membranes. 20% of polar lipids in plants represents DGDG, a bilayer-forming galactolipid. The ratio between MGDG and DGDG is important for chloroplast ultrastructure, especially during response to stress conditions.

Galactolipids are built from glycerol backbone, two long fatty acid chains, and galactose, digalactose or sulfoquinovose unit. They are not charged at physiological pH and thus represent the only neutral membrane lipid class in thylakoids. Galactolipids are synthesized exclusively at the chloroplast envelopes and from there transported to the thylakoids and extraplastidic membranes (Bruce, 1998, Dörmann and Benning, 2002). UDP-galactose serves as a water-soluble donor of galactose unit to the hydrophobic receptor diacylglycerol, DAG,

in a reaction catalyzed by MGDG synthase (Ongun and Mudd, 1968). MGDG synthase belongs to a heterogenous family (Maréchal et al., 2000) consisting of type A (atMGD1 from *Arabidopsis*, csMGD1 from *Cucumis sativa*, soMGD1 from *Spinacia oleracea*), type B (atMGD2) and type C (atMGD3) proteins. Type A MGDG synthases are expressed in all tissues, found associated in most cases with the inner envelope and represent the most important MGDG synthase in green tissues (Jarvis et al., 2000). Type B is expressed in flowers and type C in roots and young leaves (Kobayashi et al., 2004). Both B and C are found to be associated with the outer envelope membrane (Awai et al., 2001). Despite the high abundance of MGDG in plastidic membranes, MGDG synthase represents only 1/1000 of membrane proteins (Joyard et al., 1998), which makes its purification, as well as most of enzymatic analyses, almost impossible (Maréchal et al., 1994). It is a basic protein with an isoelectrical point of 9.5, active as a 45-48 kDa homodimer and is proposed to be weakly attached to the envelope membranes by electrostatic interactions (Shimojima et al., 1997).

The localization of MGDG synthase activity within envelope membranes is still a matter of controversy and seems to be different in 16:3 (*Arabidopsis*, potato, tobacco, rape, spinach) and 18:3 (pea, cucumber, barley, maize) plants (Miège et al., 1999, Jarvis et al., 2000, Ohta et al., 2000, Awai et al., 2001). In spinach, the activity was found essentially in envelope fractions enriched in the inner membrane (Tietje and Heins, 1998), whereas the situation is less clear in 18:3 plants. For instance, in pea chloroplasts, Cline and Keegstra (1983) localized the activity to the outer envelope membrane, but in several other analyses both inner and outer membranes seemed to contain significant MGDG synthase activity (Tietje and Heinz, 1998). MGDG synthase is nuclear-encoded in higher plants and no homology with eukaryotic glycosyltransferases has been found in database searches (Ohta et al., 2000). MGDG synthase is very well enzymatically characterized (Maréchal et al., 1994, Maréchal et al., 1995), but its import into chloroplasts has not been investigated yet. It has been proposed to be located in the intermembrane space of chloroplasts, associated with either outer or inner chloroplast envelope. The difference in localization between 18:3 and 16:3 plants was proposed and a lot of questions concearning its topology remained unanswered. That was the reason to investigate topology of MGDG synthase in *P. sativum* and import properties of *A. thaliana* atMGD1. AtMGD1 is assumed to be located on the outer side of the inner envelope (Benning and Ohta, 2005). Because MGD1 is proposed to be an intermembrane space protein, its import properties should be compared to those of Tic22.

4 Materials

4.1 Chemicals

All chemicals were purchased from Sigma Aldrich (München, Germany), Roth GmbH & Co. (Karlsruhe, Germany), Merck (Darmstadt, Germany), Serva Feinbiochemica (Heidelberg, Germany), Fluka Chemie AG (Buchs, Swiss), Biomol Feinchemikalien GmbH (Hamburg, Germany) and Applichem (Darmstadt, Germany).
Nitrocellulose membrane was purchased from Protran (Schleicher&Schüll, Germany), blotting-papers from Macherey Nagel (Düren, Germany), Ni-NTA Superflow column was from Qiagen (Hilden, Germany), ATP-agarose (A9264) from Sigma and nProtein A-Sepharose CL-4B from Amersham Biosciences (Uppsala, Sweden). Kodak Biomax-MR films were provided by Eastman Kodak Company (Heidelberg, Germany). Fuji film imaging plates were used for imaging analysis. They were provided by Fuji photo film company, Japan.

4.2 Enzymes and kits

Restriction enzymes, DNA- and RNA-polymerase, and other nucleic acids modifying enzymes were supplied by Roche (Mannheim, Germany), MBI Fermentas (St. Leon-Rot, Germany), Pharmacia Biotech (Freiburg, Germany) and Sigma. T4-DNA ligase was purchased from Eppendorf (Hamburg, Germany). DSP crosslinker was supplied by Pierce (München, Germany). Thermolysin was supplied by Merck, apyrase and trypsin by Sigma, soybean trypsin inhibitor and hexokinase by Roche, RNase by Amersham Pharmacia Biotech and lysozyme by Serva.
For small scale plasmid DNA isolation FastPlasmid$_{TM}$ Mini (Eppendorf) or mini boiling DNA prep were used. Large scale DNA isolation was performed with Nucleobond AX (Macherey-Nagel). Purification of DNA-fragments from agarose gels was performed using Nucleospin Extract II (Macherey-Nagel) and purification of PCR products was carried out using QIAquick PCR Purification Kit provided by Qiagen. DNA sequencing was carried out using BigDye Terminator Cycle Sequencing Ready Reaction Kit supplied by Perkin Elmer (Weiterstadt, Germany). *In vitro* transcription was performed using chemicals from MBI

Fermentas. *In vitro* translation was done with Flexi Rabbit Reticulocyte Lysate System or with TNT Coupled Reticulocyte Lysate System, supplied by Promega (Madison, USA).

4.3 Molecular weight and size markers

Protein weight standards MW-SDS-70L and MW-SDS-200 from Sigma were used for SDS-PAGE. DNA fragments site marker for agarose gels was prepared by EcoRI/HindIII restriction of λ–phage DNA, provided by MBI Fermentas.

4.4 Clones

4.4.1 Tic110

Tic110, the component of the translocon of the inner envelope membrane of chloroplasts that was used for this research derives from *Pisum sativum* (Kessler and Blobel, 1996, Lübeck et al, 1996). The coding sequence is 2991 bp long, from which first 111 bp represent transit sequence of 3.9 kDa, responsible for chloroplast targeting, and the remaining 2880 bp make 101.7 kDa mature protein. For the purpose of enhancing the radioactive signal after *in vitro* translation, additional 6 methionines were cloned at the C-terminal end of the protein. Tic110 sequence was cloned into pET21d vector.

4.4.2 pSSU-Tic110N-mSSU

The hybrid construct pSSU-Tic100N-mSSU (Lübeck et al., 1997) contains the presequence of pSSU (small subunit of RubisCO, 1-192 bp), the N-terminal part of Tic110 (112-817 bp of original clone) and the mature part of SSU (175-564 bp of the original clone). Tic110 sequence originates from *Pisum sativum* and pSSU from *Nicotiana sylvestris* (locus NSRUBSSU, Acc. No. X53426, Jamet et al., 1991). pS-110N-mSSU construct is 1257 bp long, encoding the 46 kDa protein. For the purpose of enhancing the radioactive signal after *in vitro* translation, additional 6 methionines were cloned at the C-terminal end of the protein. pS-110N-SSU sequence was cloned into pET21d vector.

MASLVLSSAAVATRSNVAQANMVAPFTGLKSAASFPVSRKQNLDITSIAS
NGGRVQCMQVWPPSSDTNNPASSSSPPQRPPKELNGIEILVDKLSSPARL
ATSAVIVAGAVAAGYGLGSRFGGSRNAALGGAVALGAAGGAAAYALNAAA
PQVAAVNLHNYVAGFDDPSILTREDIEVIANKYGVSKQDEAFKAEICDIY
SEFGSSVIPPGGEELKGDEVDKIVNFKSSLGLDDPDAAAVHMEIGRKLFR
QKLEVGDREGGVEQRRAFQKLIYVSNIVFGDASSFLLPWKRVFKV**MQVWP**
PINKKKYETFSYLPDLSQEQLLSEVEYLLKNGWVPCLEFETEHGFVYREN
NKSPGYYDGRYWTMWKLPMFGCTDATQVLAEVEEAKKAYPQAWIRIIGFD
NVRQVQCISFIAYKPEGY*MMMMMM*

Figure 3. Protein sequence of pS-110N-SSU The presequence and the mature part belonging to pSSU from *Nicotiana tabaccum* are shown in bold. The middle part of the sequence consists of the N-terminal part of Tic110 (amino acids 38-216). The presequence of the pSSU, as well as of the whole pS-110N-SSU construct, is shown in gray. Underlined are repeating sequences belonging to mSSU. Addition of six methionines at the C-terminus is shown in italic.

4.4.3 pSSU

Precursor of the small subunit of RubisCO (pSSU) was used as a control protein for various experiments performed in this thesis. The presequence of this clone originates from soybean *Glycine max* and mature part from *Pisum sativum*. The total length of this clone is 537 bp that encode corresponding 19 kDa protein (with 165 bp long presequence). The sequence was cloned into pSP64 vector.

4.4.4 Tic22

Tic22/pET21d clone used for this work derives from *A. thaliana*. The sequence used corresponds to the locus *At4g33350*. 807 bp long coding sequence gives rise to 28.5 kDa big protein, from which 177 bp or 6.3 kDa belong to the chloroplast targeting presequence. For the purpose of import experiments mature form of Tic22 (mTic22, 630 bp, 22.3 kDa) and clone with C-terminal deletion (Tic22ΔC, 675 bp, 23.9 kDa) were produced. All 3 clones were used in pET21d or pSP65 vectors.

4.4.5 MGD1

U16087 cDNA in pUNI51 vector has been obtained from the Arabidopsis Biological Resource Center. It corresponds to the *Arabidopsis* locus *At4g31780* (UGT81A1), encoding for type A MGDG synthase (AtMGD1). Coding sequence is 1602 bp long, encodes for 533 amino acids, or 56.5 kDa protein. For the purpose of overexpression and *in vitro* transcription, translation and import experiments, original clone was recloned into pET21d and pSP65 vectors. Also, for the purpose of successful overexpression, N-terminal 321 bp of the preprotein have been removed and MGD1-P clone was created, 1281 bp or 45.3 kDa long.

4.4.6 pOE33 and mOE33

Oxygen evolving complex protein of 33 kDa (pOE33) and its mature form (mOE33) were used for import competition experiments (sequence published by Murata et al., 1987) and the mature form was cloned for the purpose of this study. pOE33 was cloned into pET3c vector and is 990 bp long. mOE33 was cloned into pET21c vector and consists of 747 bp.

4.4.7 Toc34ΔTM

Toc34ΔTM used for testing the interaction with precursor proteins was from *P. sativum* (Jelic et al., 2002). It contains only 252 N-terminal amino acids, while the C-terminal transmembrane domain is removed.

4.4.8 Hsp93

Hsp93/pET21c DNA used for overexpression and antibody production was kindly provided by Prof. Dr. John E. Froehlich, Michigan State University, East Lansing, MI, USA.

4.5 Primers

All DNA primers used in PCR reactions were ordered either from MWG-Biotech (Ebersberg, Germany), Qiagen or Invitrogen GmbH (Karlsruhe, Germany).

For cloning of Tic110 into pSP65 vector:
Tic110R 5´-CCC GGG GTC GAC CTA GAA TAC AAA CTT CTC TTC CTC-3´
Tic110L 5´-CCC GGG GAA TTC ATG AAC CCT TCC ACG CTA AAA C-3´

For adding 6 Met on the C-terminal end of Tic110 and pSSU-Tic110N-mSSU in pET21d vector:
STSpETNcoFor 5´-CCC GGG CCA TGG CTT CCT TAG TTC-3´
Tic110pETNcoFor 5´-CCC GGG CCA TGG ACC CTT CCA C-3´
STSMetXhoRev 5´-CCGG CTC GAG TTA $(CAT)_6$ GTA GCC TTC GGG CTT GTA-3´
Tic110MetXhoRev 5´-CCGG CTC GAG CTA $(CAT)_6$ GAA TAC AAA CTT CTC TTC C-3´

For cloning of Tic22, mTic22 and Tic22ΔC into pET21d vector:
AraTic22NcoFor 5´-CATG CC ATG GAG TCA TCA GTG AAA CCC-3´

AramTic22NcoFor 5´-CATG CCA TGG ATG CAA TCC AAG TCT GGA ACC CC-3´
AraTic22XhoRev 5´-CATG CTC GAG ACT CTT TGA TCA AAT CCT GC-3´
AraTic22ΔCXhoRev 5´-CATG CTC GAG AAA TTT GTT GAT CTC CTC TTG ATG-3´

For cloning of Tic22, mTic22 and Tic22ΔC into pSP65 vector:
AraTic22pSP65SalFor 5´-CTAG GTC GAC ATG GAG TCA TCA GTG AAA CCC-3´
AraTic22pSP65SalRev 5´-CTAG CTG CAG TTA CTC TTT GAT CAA ATC CTG C-3´
AramTic22pSP65SalFor 5´-CTAG GTC GAC ATG CAA TCC AAG TCT GGA ACC CC-3´
AraTic22ΔCpSP65PstRev 5´-CTAG CTG CAG TTA AAT TTG TTG ATC TCC TCT GAT G-3´

For recloning of MGD1 and MGD1-P from pUNI51 to pET21d and pSP65 vectors:
AtMGD121NheFor 5´-CCC GGG GCT AGC ATG CAA ACC CTT CAA CGG-3´
AtMGD121SacRev 5´-CCC GGG GAG CTC GGC AGT GCA AGA GAG TTG-3´
AtMGD1-P21NheFor 5´-CCC GGG GCT AGC GTC GGA TTA TCG AGT GAT G-3´
AtMGD165SacFor 5´-CCC GGG GAG CTC ATG CAA ACC CTT CAA CGG-3´
AtMGD165PstRev 5´-CCC GGG CTG CAG TTA GGC AGT GCA AGA GAG-3´

For cloning of mOE33 from pOE33 in pET21c vector into pET21d vector:
mOE33NcoFor 5´-CTAG CC ATG GAA GGT GCT CCA AAG AG-3´
mOE33XhoRev 5´-GGTG CTC GAG TTC AAG C-3´

4.6 Vectors

Purpose	**Vector name**	**Company**	**Reference**
Translation vectors	pSP64	Promega	Melton et al., 1984
	pSP65	Promega	Melton et al., 1984
Overexpression vectors	pET-21c(+)	Novagen (Madison, USA)	Studier and Moffat, 1986
	pET-21d(+)	Novagen (Madison, USA)	Studier and Moffat, 1986

4.7 *E. coli* strains

Strain name	Purpose	Company	Author
DH5α	Cloning	GibcoBRL (Eggenstein, Germany)	Woodcock et al., 1989
BL21(DE3)	Overexpression	Novagen (Madison, USA)	Studier and Moffat, 1986
BL21(DE3) pRosetta	Overexpression	Novagen (Madison, USA)	Studier and Moffat, 1986

4.8 Growth media

For the purpose of overexpression of proteins and growth of transformed bacteria for the cloning all *E. coli* strains were grown in LB medium (Sambrook et al., 1989).

4.9 Radioisotopes

^{35}S-Methionine/Cysteine mixture and ^{35}S-Cysteine with specific activity of 1000 Ci/mmol were provided from Amersham Biosciences (Freiburg, Germany).

4.10 Antibodies

Primary polyclonal antibodies (α-Toc159, α-Toc75(III), α-Toc75(V), α-Toc34, α-Tic110, α-Tic22 and α-OEP16) were generated in the laboratory of Prof. Dr. Jürgen Soll by injection of purified antigens into a rabbit. α-Cpn60 antibody was kindly provided by Prof. Dr. Ulrich Hartl vom Max-Planck-Institut für Biochemie (München, Germany). Antibodies α-MGD1-P, α-Hsp93 and α-Hsp70 were produced for the purpose of this thesis (see Methods) by Pineda Antibody Service (Berlin, Germany). Secondary antibodies (goat anti-rabbit alkaline phosphatase conjugate) were obtained from Sigma.

4.11 Plant material and growth conditions

Pisum sativum (sort "Arvica", Praha, Czech Republik) was grown on vermiculit or on sand under 12 h day / 12 h night cycle in a climate chamber, at 20°C.

Spinacea oleracea was bought on the local market, kept on cold and in the dark until use.

5 Methods

5.1 General molecular biology methods

5.1.1 Standard methods

Bacterial strain culturing and preparation of glycerol stocks were performed according to standard protocols (Sambrook et al., 1989). Competent cells for DNA transformation were prepared according to Chung et al. (1989). Transformation of bacterial DH5α and BL21(DE3) strains was performed according to Pope and Kent (1996).

5.1.2 Plasmid DNA isolation

Isolation of plasmid DNA from 3 ml culture for restriction analysis, subcloning, re-transformation into another bacterial strain and sequence analysis were all adapted from the methods of Holmes and Quigley (1981). Large amounts of DNA for *in vitro* transcription and translation were isolated from 200 ml bacterial cultures by NucleobondAX kit supplied by Macherey-Nagel, according to the manufacturer's instructions. For isolation of high purity-DNA for *in vitro* transcription CsCl density gradient separation (equilibrium centrifugation based on the different intercalation efficiency of ethidium-bromide) was used (Sambrook et al., 1989). Fast purification of restricted plasmid DNA was performed by phenol/chloroform DNA extraction followed by ethanol or isopropanol precipitation (Sambrook et al., 1989) or by QiaQuick PCR Purification Kit from Qiagen.

5.1.3 Polymerase chain reaction (PCR)

The restriction sites for cloning of DNA fragments into plasmid vectors were added by the polymerase chain reaction (Saiki et al., 1998). A standard PCR reaction was carried out as recommended by the polymerase supplier (TripleMaster PCR System, Eppendorf, Hamburg, Germany). Temperatures were adjusted corresponding to the annealing temperatures of the primers. The recombinant PCR technique (Higuchi, 1990) was used in creating the DNA coding for chimerical protein constructs. Fragments of the constructs were synthesized separately in standard PCR reactions, purified over agarose gels by QiaQuick PCR Purification Kit and used as templates for the second round of PCR with the two outer primers, resulting in complete recombinant constructs (Sambrook et al., 1989). Vectors were

dephosphorylated using alkaline phosphatase from calf intestine (Roche Diagnostics GmbH, Mannheim, Germany), prior to ligation with corresponding inserts.

5.1.4 Cloning techniques

Plasmid DNA isolation, restriction of plasmid DNA and PCR-amplified fragments, ligation, as well as agarose gel electrophoresis of DNA were performed according to standard procedures (Sambrook et al., 1989). The reaction conditions for the enzymes were adjusted according to the protocols provided by the manufacturers. Standard techniques were applied for the ligation of Tic22, mTic22, Tic22ΔC and MGD1 into pET21d and pSP65 vectors, and MGD1-P, as well as Tic110 and pS-110N-SSU with additional six C-terminal methionines into pET21d vector.

5.1.5 *In vitro* transcription and translation

For *in vitro* transcription, isolated plasmid DNA was first purified either on CsCl gradient and precipitated with isopropanol, or the combination of DNA-isolation kit NucleobondAX and PCR-purification kit was used. The final pellet was resuspended in RNase free water, or the last elution was performed in water or 0.1% TAE buffer. *In vitro* transcription of linearized plasmids was carried out in a reaction volume of 50 µl containing transcription buffer (supplied by MBI Fermentas), 10 mM DTT, 100 U RNase inhibitor, 0.05% (w/v) BSA, 0.5 mM ATP, CTP and UTP, 0.375 mM m^7-Guanosine (5') ppp (5') Guanosine (cap), 10 U SP6 or T7 RNA polymerase and 2.5-3.0 µg linearized plasmid DNA. The reaction mixture was incubated for 30 minutes at 37°C to yield RNA with cap at the 5'-end. Finally, 1.2 mM GTP was added and transcription mixture was incubated for another 2 hours. mRNA was either used directly for *in vitro* translation or stored under liquid N_2. *In vitro* translation was carried out using the Flexi Rabbit Reticulocyte Lysate System or the TNT Coupled Reticulocyte Lysate System, following the manufacturer's instructions, with optimal RNA concentration and adjusted potassium acetate, magnesium acetate and DTT concentrations, which were determined by test translations. 143 µCi of ^{35}S-methionine/cysteine mixture or 100 µCi of >90% pure ^{35}S-cysteine (for the translation of Tic22) were added for radioactive labeling. After translation, the reaction mixture was centrifuged at 50,000xg for 20 minutes at 4°C and the postribosomal supernatant was used for import experiments.

Tic110 clone in pET21d vector (T7 polymerase) failed to translate successfully and that was the reason for recloning of Tic110 cDNA into a pSP65 vector, under the promoter of SP6 polymerase. Subsequent optimization did not result in any improvement in final intensity of

radioactively labelled translation product. Finally, six additional methionines at the end of Tic110 coding sequence were added, to enhance the radioactive signal of ^{35}S-labelled methionines after *in vitro* translation (Figure 3).

5.2 Isolation of chloroplasts

5.2.1 Isolation of intact chloroplasts from pea

For isolation of intact chloroplasts (Schindler et al., 1987) pea seedlings grown for 9-11 days on vermiculit, under 12/12 hours dark/light cycle were used. All procedures were carried out at 4°C. About 200 g of pea leaves were grinded in a kitchen blender in approximately 300 ml isolation medium (330 mM sorbit, 20 mM MOPS, 13 mM Tris, 3 mM $MgCl_2$, 0.1% (w/v) BSA) and filtered through four layers of mull and one layer of gauze (30 µm pore size). The filtrate was centrifuged for 1 minute at 1500xg and the pellet was gently resuspended in about 1ml wash medium (330 mM sorbit, 50 mM HEPES/KOH, pH 7.6, 3 mM $MgCl_2$). Intact chloroplasts were reisolated via a discontinuous Percoll gradient of 40% and 80% (in 330 mM sorbit, 50 mM HEPES/KOH, pH 7.6) and centrifuged for 5 minutes at 3000xg in a swing out rotor. After centrifugation two green bands of chloroplasts appeared, where the bottom one consisted of intact chloroplasts. This band was taken and washed two times, and finally resuspended in a suitable volume of wash medium. Samples of chloroplasts (5µl) were resolved in 5 ml of 80% acetone and chlorophyll concentration was estimated by measuring the optical density at three wavelengths against the solvent (Arnon, 1949). Chloroplasts were used for further import experiments.

5.2.2 Isolation of intact chloroplasts from spinach

For isolation of intact spinach chloroplasts, plants were bought at the local market few hours before isolation and kept cool and in the dark. Chloroplasts were isolated using the previously described protocol for pea chloroplast isolation.

5.3 Preparation of inner and outer envelope vesicles

For isolation of inner and outer envelope vesicles from chloroplasts pea seedlings grown for 9-11 days on sand, under 12/12 hours dark/light cycle, were used. All procedures were carried

out at 4°C. A few kg of pea leaves were grinded in a kitchen blender in 10-15 l isolation medium (330 mM sorbit, 20 mM MOPS, 13 mM Tris, 0.1 mM $MgCl_2$, 0.02% (w/v) BSA) and filtered through four layers of mull and one layer of gauze (30 µm pore size). The filtrate was centrifuged for 5 minutes at 1500xg and the pellet was gently resuspended with brush and intact chloroplasts were reisolated via a discontinuous Percoll gradient of 40% and 80%. Intact chloroplasts were washed twice with wash medium (330 mM sorbit pH 7.6), homogenized and further treated according to the modification (Waegemann et al., 1992) of the previously described method (Keegstra and Yousif, 1986).

5.4 Extraction of proteins from envelope vesicles

To distinguish between integral membrane proteins and soluble or peripheral membrane proteins, envelope vesicles (section 5.3), were pelleted at 265,000xg for 10 minutes at 4°C and resuspended in either 10 mM HEPES/KOH pH 7.6 solution for 30 minutes on ice, or 6M urea in 10 mM HEPES/KOH pH 7.6, 0.1 M Na_2CO_3 pH 11.5 or 1M NaCl for 20 minutes at RT, followed by centrifugation at 265,000xg for 10 minutes at 4°C. Separated membrane and soluble fractions were analyzed by SDS-PAGE and western-blotting.

5.5 Treatments of chloroplasts before import

5.5.1 ATP depletion from chloroplasts and *in vitro* translation product

Prior to chloroplast isolation, the peas were at least 1 hour in the dark (mostly over night). After isolation, intact chloroplasts were left on ice in the dark for 30 minutes in order to deplete ATP and therefore allow subsequent import experiments to be influenced only by exogenously added ATP as an energy source. For radioactively labelled *in vitro* translation product the ATP-hydrolysing enzyme apyrase was used to deplete endogenous ATP. A standard reaction mixture included 10 µl of translation product and 0.5 U apyrase. The reaction mixture was incubated at 25°C for 15 minutes and then used directly for import experiments. For depleting the ATP from *in vitro* translation product Micro Bio-Spin Chromatography Columns (Bio-Rad, Hercules, CA, USA) were used as well, according to producer`s recommendation. Reaction of phosphate transfer from ATP to glucose, catalyzed by hexokinase was the third method used for ATP depletion from translation product.

Supposing that the ATP concentration in reticulocyte lysate fluctuates around 10 µM, the exact amount of hexokinase needed for the complete removal of ATP could be calculated. Reaction was performed on 30°C for 10-15 minutes and stopped by addition of excess of competitive inhibitor xylose for 5 minutes at 30°C.

5.5.2 Protease pretreatment of isolated intact chloroplasts

Protease treatment of chloroplasts, prior to import of radioactively labelled protein, was carried out using chloroplasts corresponding to 1 mg chlorophyll, 1 mg thermolysin, and 0.5 mM $CaCl_2$. Wash medium (330 mM sorbit, 50 mM HEPES/KOH, pH 7.6, 3 mM $MgCl_2$) was added to the final volume of 1 ml and the reaction was incubated for 30 minutes on ice. To stop the protease action, 5 mM EDTA were added and intact chloroplasts were reisolated via a discontinuous Percoll gradient containing 5 mM EDTA and washed twice as described before.

5.6 Import Experiments

5.6.1 Import of radioactively labelled proteins into intact chloroplasts

^{35}S-labelled precursor proteins (translation products) in the maximal amount of 10% (v/v) in the reaction were mixed with freshly prepared intact pea chloroplasts (equivalent to 15-20 µg chlorophyll) in import buffer (330 mM sorbit, 50 mM HEPES/KOH pH 7.6, 3 mM $MgSO_4$, 10 mM Met, 10 mM Cys, 20 mM K-gluconate, 10 mM $NaHCO_3$, 2% BSA (w/v)) and up to 3 mM ATP in a final volume of 100 µl (Waegemann and Soll, 1995). Optionally 80 mM K_iPO_4 were added to enhance import rate (Hirsch and Soll, 1995). The import mix was incubated at 25°C for up to 20 minutes, depending on experimental requirements. Chloroplasts were reisolated over a 40% Percoll cushion, washed, and samples were separated by SDS-PAGE. Resulting gels were fluorographed (Bonner and Laskey, 1974) if needed, dried and laid on x-ray sensitive films over night.

5.6.1.1 Pulse-chase experiments

During import into chloroplasts it is possible to track changes in localization and quantity of a protein at different times, to the point it reaches its final destination. For this purpose radioactively labelled precursor protein was added to chloroplasts corresponding to 20 µg chlorophyll in the import mixture without ATP (see chapter 5.6.1) and to final volume of

100 µl. For MGD1 80 mM K_iPO_4 were added to enhance import. Samples prepared this way were incubated for 2 minutes on ice to accomplish the binding to the import receptors on the chloroplast surface. Samples were centrifuged at 1500xg for 1 minute, washed once in the import buffer and the final pellet was resuspended in import buffer containing 3 mM ATP to allow complete import. The import reactions were performed from 0 up to 32 minutes at 25ºC. Reactions were stopped after different times by addition of Laemmli buffer and samples were analyzed by SDS-PAGE.

For chasing the soluble stromal intermediates, Tic110 and pS-110N-SSU were incubated with 20 µg chloroplasts in the import mix containing 3 mM ATP for 2 minutes at 25ºC. Chloroplasts were pelleted and resuspended in the new import mix without ATP, containing 0.5 µg thermolysin per µg chlorophyll. The reaction was kept on ice for 10 minutes. Afterwards, the chloroplasts were reisolated on a Percoll cushion and resuspended in the import mix in the presence or the absence of 3 mM ATP. Samples were kept for 5 minutes on ice and imported for 10 minutes at 25ºC. All described steps were performed in the dark to minimize the generation of internal ATP, produced inside chloroplasts via photophosphorylation process. After import reaction, chloroplasts were pelleted 1 min at 1500xg, washed and separated into soluble and membrane fractions (section 5.6.2.1).

For the purpose of tracking the energy requirement of Tic110 and pS-110N-SSU on reexport import pathway radioactively labelled precursors were incubated with 20 µg chloroplasts in the import mix containing 3 mM ATP for 2 minutes at 25ºC. Afterwards chloroplasts were pelleted and resuspended in the new import mix containing either ATP, GTP or PEP at the final concentration of 3 mM, or non-hydrolyzable ATP homologs AMP-PNP and/or ATP-γ-S, at the final concentration of 5 mM. Fresh import mix was incubated on ice for 5 minutes and subsequent 10 minutes on 25ºC. All incubations were performed in the dark to diminish the influence of internally produced ATP. Afterwards, chloroplasts were pelleted 1 min at 1500xg, washed, and separated into soluble and membrane fractions.

5.6.1.2 Competition with pOE33 and mOE33 proteins

Up to 10 µM of purified competitor protein pOE33, as well as its mature form mOE33 (see section 5.10.3) were added to the import mixture. The import experiment was performed as described in section 5.6.1. Maximum 15 µg of chlorophyll per reaction was used and the import reaction lasted 5 (pSSU) to 10 or 12 minutes (Tic22, MGD1) at 25°C.

5.6.1.3 Competition for import by the cytosolic domain of Toc34 receptor GTPase

In addition to 3 mM ATP and 3 mM GTP, up to 10 µM Toc34ΔTM were added to the import mixture. First, Toc34ΔTM was preincubated with GTP in the import mixture for 10 minutes on ice. Subsequently, radioactively labelled translation product has been added for another 10 minutes to allow the interaction of preprotein with Toc34ΔTM. At the end, 15 µg of chloroplasts were added for import and the reaction was performed for 10-12 minutes at 25°C for Tic22 and MGD1 and 5 minutes for pSSU.

5.6.1.4 Inhibition of import by Ni^{2+} ions

For the purpose of stopping the import reaction on the level of the outer envelope binding 1 mM $NiSO_4$ was added to the import mix (Rothen et al., 1997). Ni^{2+} ions interact with the His-tag on the C-terminus of radioactively translated proteins and inhibit their passage across the outer envelope. The alternative to allow only binding, but not the import of preproteins into chloroplasts, was the incubation of the import mixture for 5 minutes on ice.

5.6.1.5 Protease posttreatment of intact chloroplasts

After import, the progress of translocation of proteins through the outer chloroplast envelope was controlled by the treatment of intact organelles with the protease thermolysin. Chloroplasts were pelleted from the import reaction at 1500xg for 1 minute at 4°C and resuspended in 100 µl digestion buffer (330 mM sorbit, 50 mM HEPES/KOH, pH 7.6, 0.5 mM $CaCl_2$). The digestion started with the addition of thermolysin (0.5 µg per µg chlorophyll) and was incubated on ice for 20 minutes. The reaction was stopped by addition of 5 mM EDTA, chloroplasts were pelleted again and washed in the digestion buffer containing 5 mM EDTA.

5.6.2 Suborganellar localization of imported constructs

5.6.2.1 Chloroplast fractionation into soluble and insoluble fractions

To distinguish between integral membrane proteins and soluble or peripheral membrane proteins, chloroplasts reisolated after import were lysed in 10 mM HEPES/KOH pH 7.6 for 30 minutes on ice, followed by centrifugation at 265,000xg for 10 minutes at 4°C to separate the membranes from a soluble fraction.

5.6.2.2 Extraction of proteins with 6M Urea and 0.1 M Na_2CO_3 after import

After import, reisolated chloroplast were lysed (see section 5.6.2.1), pelleted at 256,000xg, and the pellet was subsequently treated with 6M urea in 10 mM HEPES/KOH pH 7.6 or 0.1 M Na_2CO_3 pH 11.5 for 20 minutes at RT. Samples were centrifuged at 256,000xg for 10 minutes at 4°C and the pellet and soluble fractions were analyzed by SDS-PAGE.

5.6.2.3 Chemical crosslinking and immunoprecipitation

After import, chloroplasts were re-isolated on a Percoll cushion, washed and chemical crosslinking was performed by incubation of chloroplasts with 0.5 mM dithiobis-succinimdyl-proprionate (DSP) in 330 mM sorbit, 50 mM HEPES/KOH pH 7.6, 0.5 mM $CaCl_2$, for 15 minutes at 4ºC. The reaction was stopped by the addition of 125 mM glycin and further incubation at 4ºC for 15 minutes. Chloroplasts were washed twice in 330 mM sorbit, 50 mM HEPES/KOH pH 7.6, 0.5 mM $CaCl_2$ and finally lysed in hypotonic buffer (20 mM HEPES/KOH pH 7.6, 5 mM EDTA) for 30 minutes on ice. A total membrane fraction was recovered by centrifugation at 256,000xg for 30 minutes. Membranes were solublized in 1% SDS (w/v), 25 mM HEPES/KOH pH 7.6, 150 mM NaCl, diluted tenfold in the above buffer in the absence of SDS, centrifuged for 2 minutes at 20,000xg and the supernatant was used for immunoprecipitation with the antisera against Toc75(III), Toc75(V), Toc34, Tic110 and OEP16. Antisera for the previously indicated proteins were incubated with membranes and 0.5% egg albumine, rotating for 1 hour at RT, followed by purification on Protein A-Sepharose. The affinity matrix was washed 3 times with 10 bead-volumes of the mentioned buffer before the elution with Laemmli sample buffer in the presence of β-mercaptoethanol to split the crosslink products.

5.7 Binding of Toc34ΔTM to precursor proteins

For one reaction 300 µg purified Toc34ΔTM protein was coupled to 10 µl Ni-NTA matrix in the binding buffer (50 mM NaCl, 50 mM Na_iPO_4, 0.5% BSA, pH 7.9) for 45 minutes, rotating at RT. Such matrix was used for investigation of interaction of soluble Toc34ΔTM with precursor proteins. The prepared matrix was preincubated with 1 mM GTP, and subsequently 10-12 µl of a radioactively labelled translation product were applied in the reaction containing 1 mM GTP, 2 mM $MgCl_2$, 20 mM Tris/HCl pH 7.6, 50 mM NaCl and 0.5% BSA. Incubation lasted 45-50 minutes, rotating at RT. The matrix was subsequently washed 3 times with wash

buffer (50 mM NaCl, 50 mM NaPi, 30 mM imidazole, pH 7.9) and eluted in 50 µl elution buffer (50 mM NaCl, 50 mM NaPi, 300 mM imidazole, pH 7.9). The flow through after binding of precursor proteins to Ni-NTA-Toc34ΔTM matrix, the last wash and the elution were analysed by SDS-PAGE and the gel was exposed on an x-ray film.

5.8 Stromal processing assay

Chloroplasts were isolated from 9-11 days old pea, as described (section 5.2.1). As isolation and wash medium 330 mM sorbit and 50 mM HEPES/KOH pH 7.5 were used. Chloroplasts corresponding to 800 µg chlorophyll were pelleted at 1500xg for 1 minute and lysed in 1 ml of 5 mM ice-cold HEPES/KOH pH 8 for 30 minutes on ice. After centrifugation at 16,000xg for 10 min on 4ºC, subsequent ultracentrifugation of the supernatant using 137,000xg for 1 hour was applied. The supernatant containing the active stromal processing peptidase was used in the processing assay. 15 µl supernatant, 2.5 µg chloramphenicol, 2-3 µl radioactively labelled translation product, and 20 mM HEPES/KOH pH 8 were mixed in a total volume of 25 µl for 90 minutes at 26ºC. The reaction was stopped by addition of Laemmli buffer and samples were analyzed by SDS-PAGE.

5.9 Isolation of stromal protein Hsp70

Intact chloroplast from two Percoll gradients (isolated as described in section 5.2.1) were washed 2 times in 330 mM sorbit, 50 mM Hepes/KOH pH 7.6, 3 mM $MgCl_2$, pelleted at 1000xg for 1 minute, gently resuspended in 15 ml buffer A (10 mM KAc, 2 mM $MgCl_2$, 2 mM DTT, 20 mM HEPES/KOH pH 7.6), 1% Triton X-100 and left for 30 minutes on ice. Subsequent centrifugation at 12,000xg for 15 min at 4°C provided non-solubilized debris in pellet and the supernatant that was diluted to 50 ml with buffer A and coupled to 65 mg ATP-agarose (washed previously with buffer A by vacuum filtering through a nitrocellulose membrane). The incubation was performed over night at 4°C. Next day the matrix was washed with 10 ml buffer A, 10 ml buffer B (1 M KAc, 2 mM $MgCl_2$, 2 mM DTT, 20 mM HEPES/KOH pH 7.6) and again 10 ml buffer A. 10 times 1 ml elution fractions were collected by applying buffer E (10 mM ATP, 10 mM $MgCl_2$, 2 mM DTT, 20 mM HEPES/KOH pH 7.6, 10 mM KAc). The procedure was repeated until a sufficient amount of

highly purified protein fractions was collected. Purified fractions were concentrated using Amicon Microcon centrifugal filter devices (Millipore Corporation, Bedford, MA, USA) and stored in 50 mM NaCl, 20 mM Tris/HCl pH 8.5 in concentration of 1 mg/ml in the purpose of antibody production.

5.10 Protein overexpression and purification

5.10.1 Overexpression and purification of Hsp93

Stromal chaperone Hsp93 DNA in pET21c vector was transformed into BL21 (DE3) pRosetta competent cells. Hsp93 protein was overexpressed in 1 l LB medium with 100 mg ampicilin, 100 mg chloramphenicol, 1 mM $MgSO_4$ and 0.4% glucose. Cells were grown at 37°C till they reached $O.D._{600}$ of 0.6, induced with 0.5 mM IPTG and incubated at 30°C for 3 to 4 hours. Bacteria were pelleted and the protein was purified under denaturing conditions. Each 500 ml bacterial culture was lysed using 30 ml lysis buffer (50 mM NaCl, 20 mM Tris/HCl, 5 mM β-MeEtOH, 8 M Urea, pH 8.0 to 8.5). Urea was previously deionized using MTO-Dowex Marathon MR-3 beads (Supelco, Bellefonte, PA, USA). Solubilized cells were stirred around 40 minutes, till the solution was translucent. The supernatant taken after centrifugation at 10,000xg for 30 minutes at RT was further filtered and purified using anion-exchange chromatography (ResourceQ and MonoQ column, Aekta, Amersham Biosciences). ResourceQ was used for large scale purification and MonoQ for obtaining highly purified fractions. The isoelectrical point for Hsp93 of 6.55 was determined *in silico*, so the pH during purification was adjusted to 8.0-8.5. After binding, protein was washed and eluted from the column by applying the linear gradient ranging from 50 mM to 700 mM NaCl in Tris/HCl pH 8. The purest protein fractions were obtained by elution with 400-550 mM NaCl. Those elutions were concentrated using MF-Millipore membrane filters with the pore size of 30 kDa, and dissolved in 50 mM NaCl in 20 mM Tris/HCl pH 8.5, at a concentration of 1 mg/ml, for the purpose of antibody production.

5.10.2 Overexpression and purification of MGD1-P

Due to the problems with expression of full length MGD1 construct, a form of this protein without presequence (MGD1-P) was used for antibody-production. MGD1-P in pET21d vector was overexpressed in BL21(DE3) pRosetta cells, in 1 l LB medium with 100 mg ampicilin, 100 mg chloramphenicol, 1 mM $MgSO_4$ and 0.4% glucose. Cells were grown at

37°C till they reached $O.D._{600}$ of 0.6, induced with 0.5 mM IPTG and incubated at 30°C for 3 hours. Bacteria were pelleted, lysed according to QIAexpressionist (Qiagen) protocol and insoluble protein was purified under denaturing conditions on Ni-NTA matrix. For each 500 ml bacterial culture 1 ml of Ni-NTA matrix was used. After applying the sample in binding buffer (QIAexpressionist), column was washed in 10 ml W1 (150 mM NaCl, 20 mM Tris/HCl, 6 M urea, pH 8), 5 ml W2 (1 M NaCl, 20 mM Tris/HCl, 6 M urea, pH 8), again 5 ml W1 and eluted with 5 times 1 ml of 150 mM NaCl, 20 mM Tris/HCl, 6 M urea, 500 mM imidazole, pH 8. Purification has been tried using pH gradient for washing (pH 6.3) and elution (pH 5.9 and 4.5). This trial did not result in satisfying results and purifications using HiTrap column and by cation-exchange chromatography using PorousS column have been performed (Äkta). Because these methods did not provide final high protein purity, subsequent purification by gel elution was applied. After SDS-PAGE, gel was washed shortly in ddH_2O, stained in 0.3 M $CuCl_2x2H_2O$ or $CuSO_4x5H_20$ for 5 minutes, the band of the corresponding size (45.3 kDa) was excised, washed few times in H_2O, 3 x 10 minutes in 250 mM EDTA, 250 mM Tris/HCl pH 9, cutted in small pieces and incubated 45 minutes at RT in 1 ml soaking buffer (125 mM Tris/HCl pH 6.8, 0.5% SDS, 1 mM EDTA) and 4 M Urea. Afterwards the gel pieces were transferred to dialysis bags (Roth) and eluted over night using electrical current of 6 mA in DNA gel-electrophoresis cell filled with Laemmli buffer in addition to 4 M Urea. Elutions were collected, concentrated using MF-Millipore membrane filters with the pore size of 30 kDa and dissolved in 50 mM NaCl in 20 mM Tris/HCl pH 8 at the concentration of 1 mg/ml for the purpose of antibody production.

5.10.3 Overexpression and purification of pOE33 and mOE33

Transformed BL21(DE3) competent cells were grown in LB medium containing 100 µg/ml ampicilin (and 1 mM $MgSO_4$ and 0.4% glucose for mOE33) till $O.D._{600}$ reached 0.6. Expression was induced by 1 mM IPTG and cells were grown for 3 hours on 37°C. Cells were harvested by centrifugation and the expressed protein was isolated in form of inclusion bodies. The protocol for purification of His-tag containing proteins under denaturing conditions and elution by changing the pH (QIAexpressionist) were applied. Refolding of the protein was accomplished by dialysis against 6, 4, 2 and 0 M urea (over night, for 4 hours, 4 hours and overnight, respectively). Aggregated (misfolded) material was pelleted by centrifugation at 27,000xg for 10 minutes at 4°C. The protein concentration in the supernatant was estimated and used for competition experiments.

5.10.4 Overexpression and purification of Toc34ΔTM

Toc34ΔTM protein was overexpressed and purified for the purpose of testing its interaction with precursor proteins prior to import into chloroplasts. The transformed BL21(DE3) cells were grown at 37°C in 200 ml LB medium containing 100 µg/ml ampicilin to $O.D._{600}$=0.6 and the expression was initiated by the addition of 1 mM IPTG. After three hours of incubation at 37°C the cells were harvested by centrifugation and the expressed protein was isolated soluble. The bacterial pellet corresponding to 200 ml liquid culture was lysed in 20 ml lysis buffer (50 mM Na_iPO_4, pH 7.9, 300 mM NaCl, 10 mM imidazole) in addition to 25 mg lysozyme, and incubated for 30 minutes on ice. Sample was sonicated 6 times 10 bursts at a middle strength, with 10 seconds of cooling period between bursts, and centrifuged at 25,000xg for 30 minutes at 4°C. Supernatant was applied on 400 µl Ni-NTA matrix, washed with 15 column volumes of the same buffer containing 30 mM imidazole and eluted by 250-300 mM imidazole. Final protein concentration was determined by the Bio-Rad method. The protein was always used fresh and diluted so that a maximum imidazole concentration in import reaction did not exceed 30 mM.

5.11 Coimmunoprecipitation of Tic110

Pea chloroplasts corresponding to 200 µg chlorophyll were pelleted at 1500xg for 1 minute at 4°C and solubilized in 200 µl IP buffer (immunoprecipitation buffer consisting of 25 mM HEPES/NaOH pH 7.6 and 150 mM NaCl) with addition of 1.5% DeMa and 0.05% egg albumine. After solubilization, the sample was diluted to 1ml with IP buffer, 0.5% egg albumine and 5 µl primary antiserum (α-Hsp70, α-Hsp93) or preimmune serum (α-preHsp70, α-preHsp93) were added and incubated rotating for 90 min at RT. 50 µl of Protein A-Sepharose matrix was washed in IP buffer containing 0.5% egg albumine and 0.3% DeMa, applied to the sample, and incubated rotating for another 90 minutes on RT. Matrix was subsequently washed 2 times with 500 µl IP buffer containing 0.3% DeMa and 1 time without DeMa. Elution was performed using Laemmli buffer, vortexing and boiling the sample at 99°C for 1 minute. Flow through, washes and elution fractions were analyzed by SDS-PAGE and western blotting, using α-Tic110 antibodies.

Coimmunoprecipitation of Tic110 and pSSU-Tic110N-mSSU soluble stromal intermediates was performed as follows. After import, intact chloroplasts were reisolated on a 40% Percoll cushion, washed, fractionated onto soluble and pellet fractions (see section

5.6.2.1). Soluble fraction was diluted 1:1 in 2xIP buffer and incubated with 5 µl of α-Hsp70, α-Hsp93, α-Cpn60 or α-OEP16 antisera for 1 hour at RT, in the presence of 0.5% egg albumine and 0.3% DeMa. Subsequent purification on Protein A-Speharose was performed as described previously.

5.12 Methods for separation and identification of proteins

5.12.1 SDS-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE)

Electrophoretic separation of proteins under denaturing conditions was performed in a discontinuous gel system (Laemmli, 1970). For separation gels 10 to 13% acrylamide was used, and for stacking gels 5% acrylamide.

5.12.2 Detection of proteins in gels

After separation of proteins on polyacrylamide gels a number of standard detection techniques were used. Staining solution containing 0.18% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) methanol and 7% (v/v) acetic acid enabled visual recognition of bands equivalent to 0.1-10 µg protein. Gels were stained for 15 minutes on a shaker and unbound dye was removed by 15-30 minutes washing in a destaining solution (40% (v/v) methanol, 7% (v/v) acetic acid, 3% (v/v) glycerol). For detection of ^{35}S-labelled proteins, acrylamide gels were dried and exposed on x-ray films (Kodak Biomax MR) underlayed with photoenhancing screens (Agfa MR800) for 1-3 days, depending on the amount of radioactivity present. To estimate the radioactivity of the samples, films were laid on imaging plates (BAS-MS) for 1 day. The plates were screened using phosphoimaging scanner FLA-3000 and band intensities were analyzed using AIDA image analyzer program for advanced image analysis (Advanced Image Data Analyzer v.3.52, 2D densitometry).

5.12.3 Western transfer

Directly following the SDS-PAGE proteins were transferred onto nitrocellulose membranes using the semi-dry electro blotting technique (Trnovsky, 1992). The gel was laid onto 6 sheets of blotting-papers, soaked in anode buffer I (0.3 M Tris pH 10.4, 20% MeOH), 4 sheets soaked in anode buffer II (25 mM Tris pH 10.4, 20% MeOH), the membrane rinsed in the same buffer and covered by 6 sheets of blotting-papers in cathode buffer (25 mM Tris pH 7.5, 4 mM aminocapronic acid and 20% MeOH). The “sandwich” was placed into transfer

apparatus chamber. The working current was 0.8 mA per cm^2 of the gel for 1 hour. The membranes were then stained with 0.5% Ponceau S in 1% acetic acid, molecular standard marker positions were marked, and the membranes subjected to further analysis.

5.12.4 Immunodecoration of proteins with antibodies

The nitrocellulose membrane with bound proteins was saturated by 3x10 minutes wash in TN (100 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.3% milk powder, 0.03% BSA) or in TTBS buffer (100 mM Tris/HCl pH 7.5, 0.9% NaCl, 1% BSA, 0.1% Tween-20). The same buffers were used for all following procedures. The membranes were sealed in polyethylene bags and incubated for 3 hours at RT or over night at 4ºC with the appropriate antiserum, diluted 1:1000. Excess primary antibodies were then removed by 3x10 minutes washing and the secondary antibody (goat anti-rabbit alkaline phosphatase conjugate) in the dilution 1:10000 was applied to the membrane and incubated for 1 hour. Staining reactions were performed in 100 mM Tris/HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.035% NBT (w/v) and 0.0175% BCIP (w/v).

5.12.5 General methods of protein biochemistry

Protein concentrations in solutions were roughly evaluated from the stained gels by comparing protein bands to protein weight standards. More precise determination of protein concentration was performed using the Bradford Bio-Rad reagens. To precipitate proteins from diluted solutions, 10% final concentration of TCA (w/v) was added to samples and incubated for 20 minutes on ice, followed by 15 minutes centrifugation at 25,000xg. Samples were neutralized by Tris-base, as demanded by experimental conditions.

5.12.6 Fluorography

Fluorography was applied for the purpose of enhancing of weak radioactive signals after the SDS-PAGE, before drying the gel and exposing it on an x-ray film. Two methods were used. After destaining, gel was incubated 4 times 10 minutes in DMSO, 30 minutes in 20% PPO in DMSO, washed 2 times 15 minutes in water prior to drying and exposure on a film.

Another approach used was washing a gel after destaining 2 times for 15 minutes in water, incubating 20 minutes with 16% (w/v) Na-salicylate and drying (without washing in water).

6 Results

6.1 Import properties of the inner membrane protein Tic110

6.1.1 Import of Tic110 and pSSU-Tic110N-mSSU

In vitro translation of Tic110 resulted in two products, one around 95 and another around 120 kDa (Figure 4A, lane 1). Translation of pS-110N-SSU resulted in a single band of around 56 kDa (Figure 4A, lane 3). Both radioactively labelled Tic110 translation products were imported into chloroplasts under the standard conditions (see section 5.6.1) and the lower product probably represents a version of Tic110 truncated at the C-terminus. The difference in size between the precursor and the mature form of the protein corresponded to the transit sequence of 3.9 kDa that was removed by the stromal processing peptidase upon import into chloroplasts (Figure 4A, lane 2). Thermolysin posttreatment of the chloroplasts removed the precursors bound to the organellar surface, leaving only protected, mature forms of proteins intact. Both mature forms of Tic110 were protected from thermolysin degradation, indicating that the mature Tic110 is localized inside chloroplasts (Figure 4A, lane 2). The hybrid construct pS-110N-SSU was imported into pea chloroplasts under the standard conditions as well. Its mature part, lacking the presequence of around 6.5 kDa, was protected from thermolysin degradation (Figure 4A, lane 4).

The pulse-chase import experiment was performed to observe the import intermediate forms and to investigate their energy requires. Import of radioactively labelled translation products pTic110 and pS-110N-SSU has been performed for 2 minutes at 25°C. Chloroplasts were pelleted and reisolated in the fresh import mix containing thermolysin in the concentration of 0.5 µg per µg chlorophyll to remove precursors just bound to the chloroplast surface. Chloroplasts were reisolated and finally incubated in the presence or the absence of 3 mM ATP. The addition of ATP was essential for the complete incorporation of soluble Tic110 and pS-110N-SSU into the membrane. Under limiting ATP conditions, only a portion of the proteins was imported into the inner envelope membrane (Figure 4B, lanes 2, 7, 11) and mostly around 20% could be observed in the soluble fraction as soluble stromal intermediates (Figure 4B, lanes 3, 8, 12). In the presence of 3 mM ATP almost the entire mature protein pool was inserted into the membrane (Figure 4B, lanes 5, 10 and 14 in

comparison to 4, 9 and 13), while the lack of an energy source arrested the soluble intermediate in the stromal compartment. These results indicated that the integration of Tic110 and pS-110N-SSU in the inner envelope is dependent on ATP.

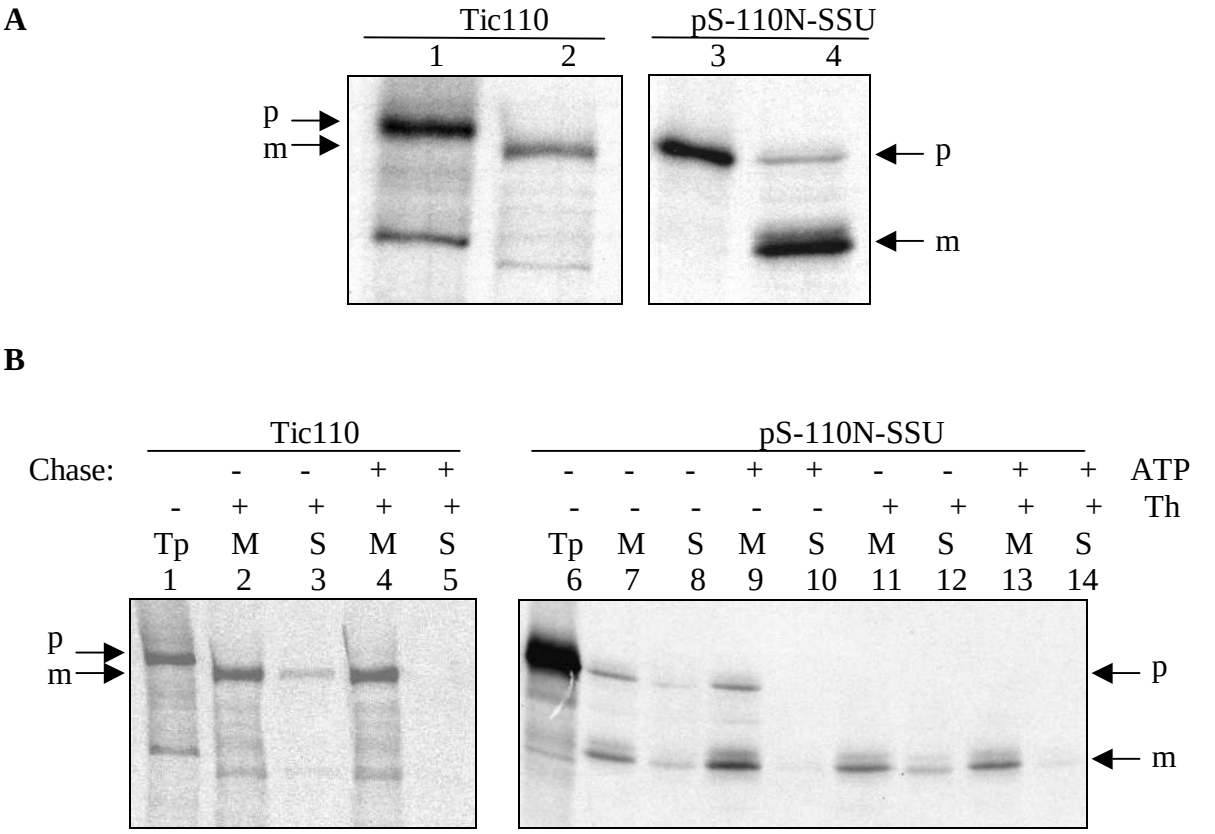


Figure 4. A. Tic110 and pS-110N-SSU import into pea chloroplasts. Import of ^{35}S-pTic110 and ^{35}S-pS-110N-SSU was performed for 15 minutes at 25ºC, in the presence of 3 mM ATP. After import, samples were treated by thermolysin (lanes 2 and 4). Lanes 1 and 3 indicate 1/10 of the translation products. **B. Chase of Tic110 and pS-110N-SSU.** ^{35}S-pTic110 and ^{35}S-pS-110N-SSU were incubated with intact pea chloroplasts for 2 minutes at 25ºC, in the presence of 3 mM ATP (pulse). After this time chloroplasts were transferred to the new import mix either in the presence of 0.5 µg thermolysin per µg chlorophyll (lanes 2-5, 11-14) or without thermolysin (lanes 7-10) for 10 minutes on ice. After this treatment, chloroplasts were reisolated on a Percoll cushion, washed and resuspended in the import mix containing 3 mM ATP (lanes 4, 5, 9, 10, 13, 14) or without ATP (lanes 2, 3, 7, 8, 11, 12) and incubated for 10 minutes at 25ºC. After import, chloroplasts were separated into the membrane (M) and soluble (S) fractions. Lanes 1 and 6 represent 1/10 of the translation product used for the import reaction. Precursor (p) and mature (m) forms of Tic110 and pS-110N-SSU are indicated by arrows.

The stromal intermediates that were observed for both imported proteins represent the mature forms: Tic110 and 110N-SSU. To confirm that the removal of their transit sequences occurs in the stroma, pTic110 and pS-110N-SSU were subjected to a stromal processing. Chloroplast stroma was isolated (section 5.8) and incubated together with the radioactively labelled

Tic110 and pS-110N-SSU for 90 minutes at 26°C. Tic110 and pS-110N-SSU were both processed by the stromal extract (Figure 5).

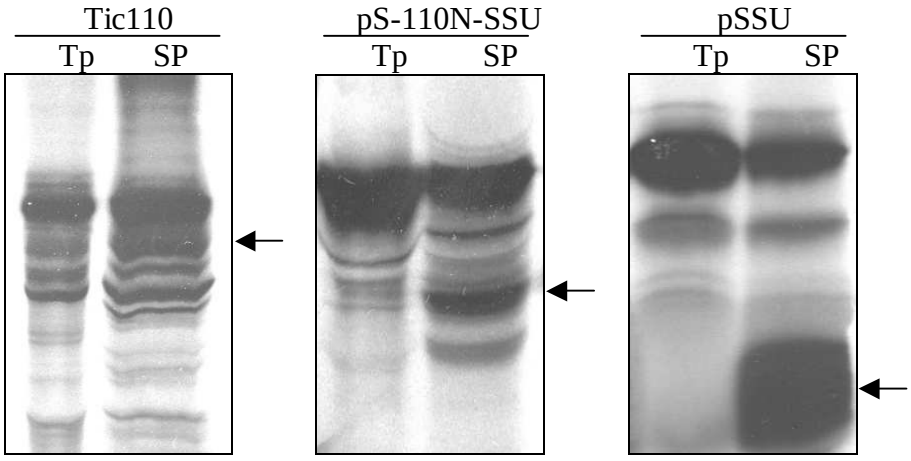


Figure 5. Processing of Tic110 and pS-110N-SSU by the stromal extract. Stroma was isolated from pea chloroplasts and incubated with radioactively labelled translation products ^{35}S-pTic110, ^{35}S-pS-110N-SSU and ^{35}S-pSSU as a control, for 90 minutes at 26°C. Reactions were stopped by addition of Laemmli buffer and samples were analyzed by SDS-PAGE. Tp indicates 2 µl of the corresponding translation product and SP 2µl of the corresponding translation product after processing. Mature forms: Tic110, 110N-SSU and SSU, appearing after the processing by the stromal processing peptidase, are indicated by arrows.

6.1.2 Soluble stromal import intermediates

In order to follow the import of Tic110 through different chloroplast compartments on a time-scale, a standard import reaction was performed in the presence of 3 mM ATP. After 0.5, 2, 5, 10 and 20 minutes the reaction was stopped by transfer to ice and reisolated chloroplasts were separated into the membrane and soluble fractions (section 5.6.2.1).

Changing the import duration led to the appearance of the stromal intermediate found in the soluble fraction, the amount of which increased during the first five minutes of import (Figure 6, lanes 5, 7, 9). After this time the concentration of the soluble intermediate in the stroma decreased (Figure 6, lanes 11, 13) because of its re-export into the membrane. Enrichment of the membrane fraction with mature proteins could be observed with time (Figure 6, lanes 4, 6, 8, 10, 12). The appearance of the soluble intermediate was observed already after 0.5 minutes of import (Figure 6, lane 5) and its ratio to the membrane-bound form increased to reach the maximum value between 2 and 5 minutes (Figure 6, lanes 7, 9). After this time, the soluble stromal intermediate progressively moved to the membrane fraction, and nearly no intermediate was observed after 20 minutes of import (Figure 6, lane

13). The described experiment enabled us to follow the import pathway of Tic110 through the chloroplast envelopes to the stroma, where its transit peptide was removed by the stromal processing peptidase (Figure 5), to the re-export into the inner envelope, to its final location.

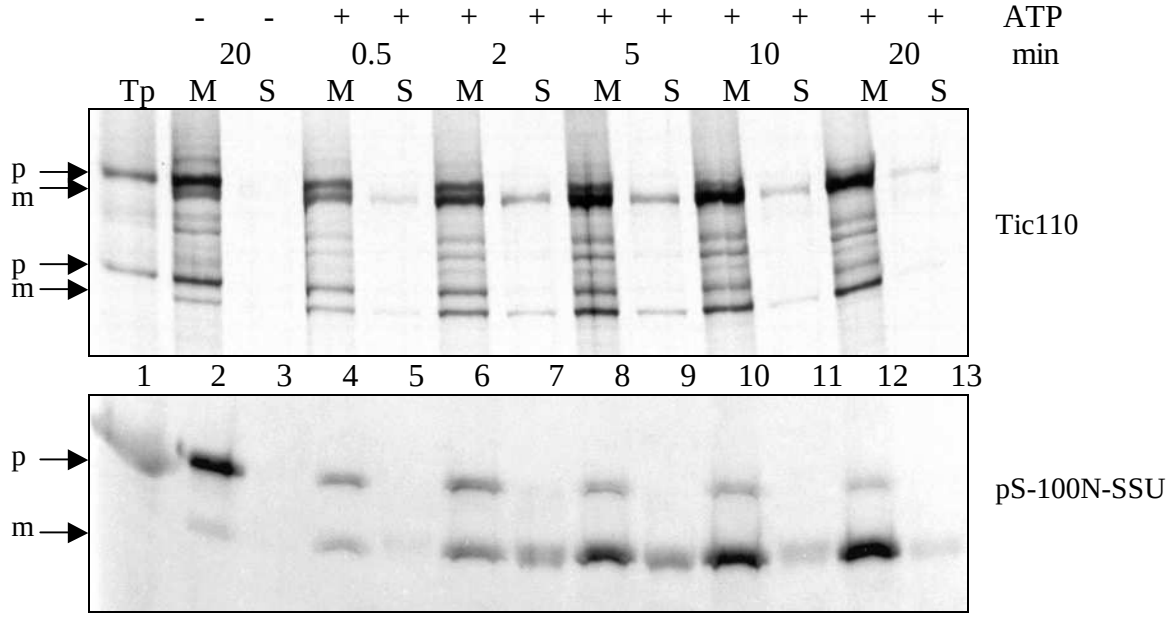


Figure 6. Time-scale import of Tic110 and pS-110N-SSU. ^{35}S-pTic110 and ^{35}S-pS-110N-SSU were incubated with intact pea chloroplasts using the standard conditions, for 0.5-20 minutes at 25ºC. 3 mM ATP (lanes 4-13) or no NTPs (lanes 2-3) were used in the import mix. After import chloroplasts were separated into the membrane (M) and soluble (S) fractions. Lane 1 indicates 1/10 of the translation product used for the import reaction. Precursor (p) and mature (m) forms of Tic110 and pS-110N-SSU are indicated by arrows.

A control experiment without the addition of ATP in the import mixture (Figure 6, lanes 2 and 3) indicates that the import into the stroma needs higher amounts of ATP. Comparing the ratio between soluble and membrane-associated forms of Tic110 on a time-scale, it appears that most of the protein was found in the membrane fraction. These results imply that the processing of Tic110 is a very fast process. The mature Tic110 visible under the ATP-depletion probably represents only the portion of Tic110 that is arrested in the inner envelope membrane translocon, rather than completely translocated to its final position. In such state the protein can still be processed by the stromal processing peptidase, by extruding its N-terminal presequence towards the stroma.

After 2 minutes of import the ratio between the soluble and the membrane-bound form of pS-110N-SSU was around one (Figure 6, lanes 6 and 7). The same ratio for Tic110 never exceeded 0.15 to 0.20. The import conditions under which the highest soluble to membrane protein ratio was obtained were considered the most suitable for further investigations of the

energy requirements of Tic110 and 110N-SSU for subsequent re-export from the stroma to the inner membrane. For this purpose, radioactively labelled precursors pTic110 and pS-110N-SSU were incubated with intact pea chloroplasts corresponding to 20 µg chlorophyll, in the import mix containing 3 mM ATP for 2 minutes at 25ºC. After 2 minutes chloroplasts were pelleted and resuspended in a new import mix containing ATP, GTP, PEP, or non-hydrolyzable ATP homologs AMP-PNP and ATP-γ-S, as described in section 5.6.1.1.

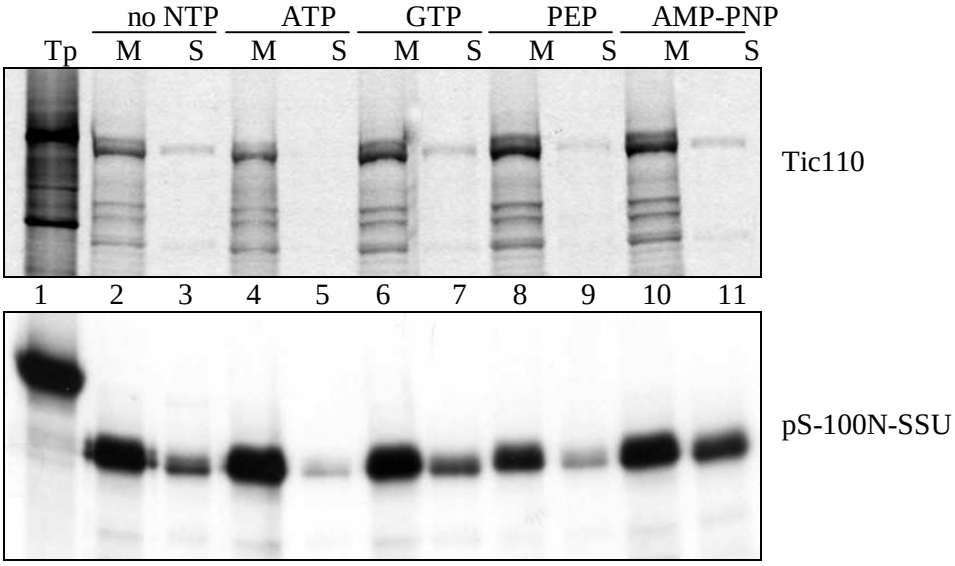


Figure 7. Energy requirement for the re-export of Tic110 and pS-110N-SSU. Both radioactively labeled proteins were imported in the standard import reaction at 25ºC in the presence of 3 mM ATP. The import was arrested after 2 minutes and the chloroplasts were transferred into a new import mix containing either no NTPs (lanes 2 and 3), 3 mM ATP (lanes 4 and 5), 3 mM GTP (lanes 6 and 7), 3 mM PEP (lanes 8 and 9) or 5 mM AMP-PNP (lanes 10-11). Samples were incubated for 5 minutes on ice followed by 10 minutes of import at 25ºC. After import chloroplasts were separated into the membrane (M) and soluble (S) fractions. Lane 1 represents 1/10 of the translation product used for the import reaction.

Using this approach the soluble stromal intermediate, visible after 2 minutes of import (Figure 6, lane 7), could be investigated. After addition of 3 mM ATP, all (Tic110) or nearly all (pS-110N-SSU) soluble intermediates were re-exported to the membrane (Figure 7, lanes 4 and 5), while in the presence of other energy sources the soluble form could be trapped to a certain extent (Figure 7, lanes 3, 7, 9, 11). The highest ratio between the soluble intermediate and the membrane fraction of the protein was observed after addition of GTP and AMP-PNP (Figure 7, lanes 7 and 11 respectively), that was especially obvious for pS-110N-SSU import. ATP-γ-S alone or in the combination with AMP-PNP provided the same result as AMP-PNP (data not shown). The observation that GTP and GMP-PNP provided the highest ratio

between the soluble and the membrane portion of the protein was used for the isolation of larger quantities of soluble stromal intermediates Tic110 and 110N-SSU for the purpose of immunoprecipitation with stromal chaperones (section 5.11). To further improve the quantity of the soluble stromal intermediate, imports were performed under different temperature, time and ATP conditions (data not shown).

Several combinations of conditions resulted in the highest soluble to membrane fraction ratio of Tic110 and 110N-SSU. 3.5 minutes of import at 25°C in the presence of 3 mM ATP were chosen for further experiments (Figure 8, lane 7). To investigate the interaction of the soluble intermediates with the stromal chaperones first the antibodies against Hsp93 and Hsp70 had to be produced.

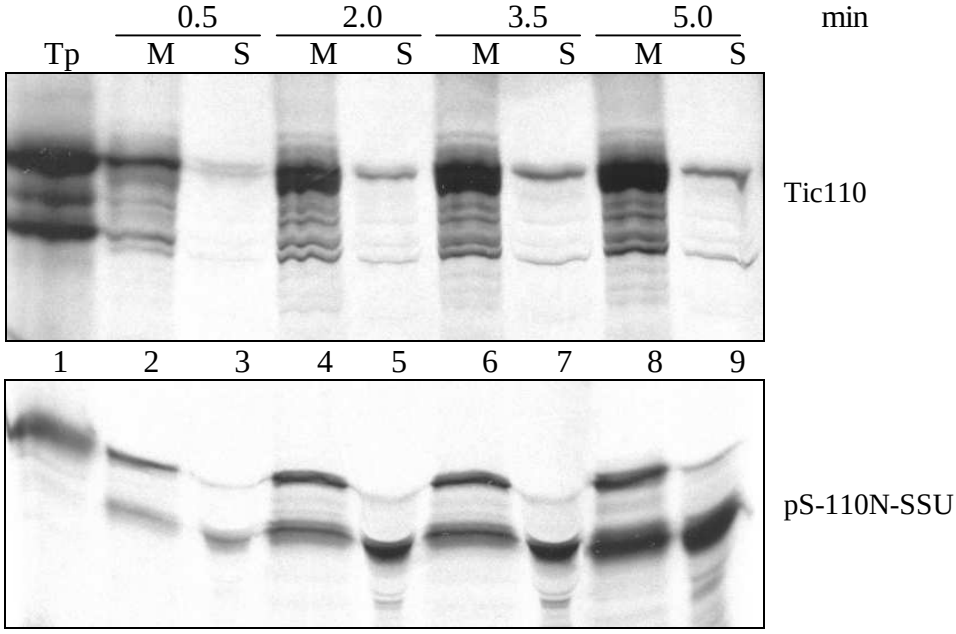


Figure 8. Import conditions used for the isolation of the soluble stromal intermediate. ^{35}S-Tic110 and ^{35}S-pS-110N-SSU were incubated with intact pea chloroplasts using standard conditions, for 0.5-5.0 minutes at 25ºC, in the presence of 3 mM ATP. After import chloroplasts were reisolated on a Percoll cushion, washed, and separated into the membrane (M) and soluble (S) fractions. Lane 1 indicates 1/10 of the translation product used for the import reaction.

6.1.3 Purification of stromal chaperone Hsp70

Stroma of *Pisum sativum* contains at least two isoforms of the Hsp70 family: proteins CSS1 and S78 (Kessler and Blobel, 1996). The population of stromal Hsp70s was isolated from pea by direct coupling of freshly isolated chloroplast stroma to an ATP-agarose matrix, as described in section 5.9. The ability of the chaperones to bind ATP was used for their

purification. Hsp70 was eluted from the ATP-agarose matrix by increased ATP concentration and collected fractions were analyzed by SDS-PAGE (Figure 9A). The resulting protein fraction was highly pure and no further purification was required (Figure 9A, B). The purified protein was concentrated to 1 mg/ml and used for antibody production. Antibodies were tested on chloroplast stroma and purified protein (Figure 9C).

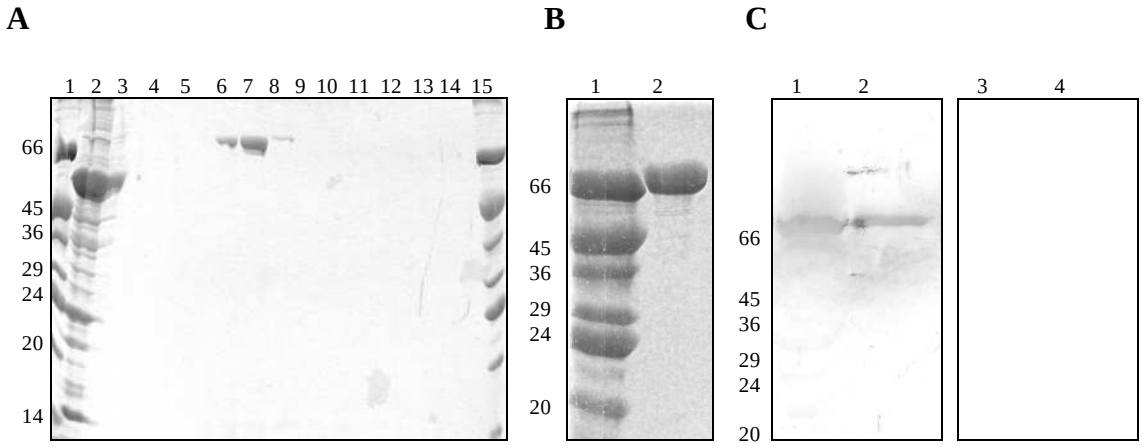


Figure 9. Purification of Hsp70 from chloroplast stroma. A. Freshly isolated stroma from pea chloroplasts was coupled to an ATP-agarose. 20 µl of low molecular weight marker is in lanes 1 and 15, 1/1000 of the flow through after coupling to the ATP-agarose is in lane 2. Lanes 3-5 represent 0.65% of washes with buffer A1, buffer B and buffer A2, respectively. Lanes 6-14 represent the elution fractions, 1/20 volume of each. **B.** Hsp70 combined from elutions 1, 2 and 3 (lanes 6-8 in A) from many purifications was concentrated to 1 mg/ml, using Amicon Microcon centrifugal filter devices (lane 2 contains 5 µl). 20 µl of low molecular weight marker is in lane 1. **C. Testing of Hsp70 antibodies produced in rabbit.** 7.5 µl of chloroplast stroma (lanes 1 and 3) and 0.5 µg of purified Hsp70 protein (lanes 2 and 4) were analyzed by SDS-PAGE and western transfer and subsequent immunodecoration with α-Hsp70 antibodies (lanes 1, 2) or with the preimmune serum (lanes 3, 4).

6.1.4 Overexpression and purification of the stromal chaperone Hsp93

Hsp93 was overexpressed under conditions described in chapter 5.10.1 (Figure 10A). Overexpressed protein was tested for solubility and its biggest portion was isolated as inclusion bodies (Figure 10B). Overexpressed Hsp93 protein was further purified under denaturing conditions. After solubilization in 8 M Urea, pH 8.0 to 8.5, the sample was centrifuged and the supernatant filtered and purified using the anion-exchange chromatography ResourceQ and MonoQ columns (Äkta, Amersham Biosciences). Absorption spectrum at 280 nm was measured and fractions of 0.5 ml corresponding to each observed peak were collected and analyzed by SDS-PAGE (Figure 11A-C).

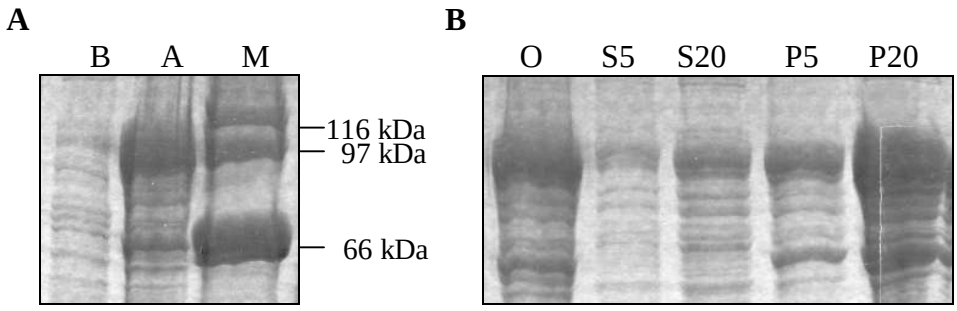


Figure 10. Overexpression of Hsp93. A. Hsp93 was overexpressed in *E. coli* BL21(DE3) pRosetta competent cells, for 3 hours at 30°C. B, sample before induction; A, sample after 3 h of expression, and M, 20 µl of high molecular weight marker. **B.** After overexpression, bacteria were lysed and pelleted. The soluble (S) and pellet (P) fractions were analyzed by SDS-PAGE. O, sample after overexpression; S5 and S20 indicate 5 and 20 µl of soluble portion of Hsp93; and P5 and P20, 5 and 20 µl of non-soluble portion of Hsp93, respectively.

Purified protein was concentrated to 1 mg/ml and used for antibody production. Antibodies were tested on chloroplast stroma and purified proteins (Figure 11D).

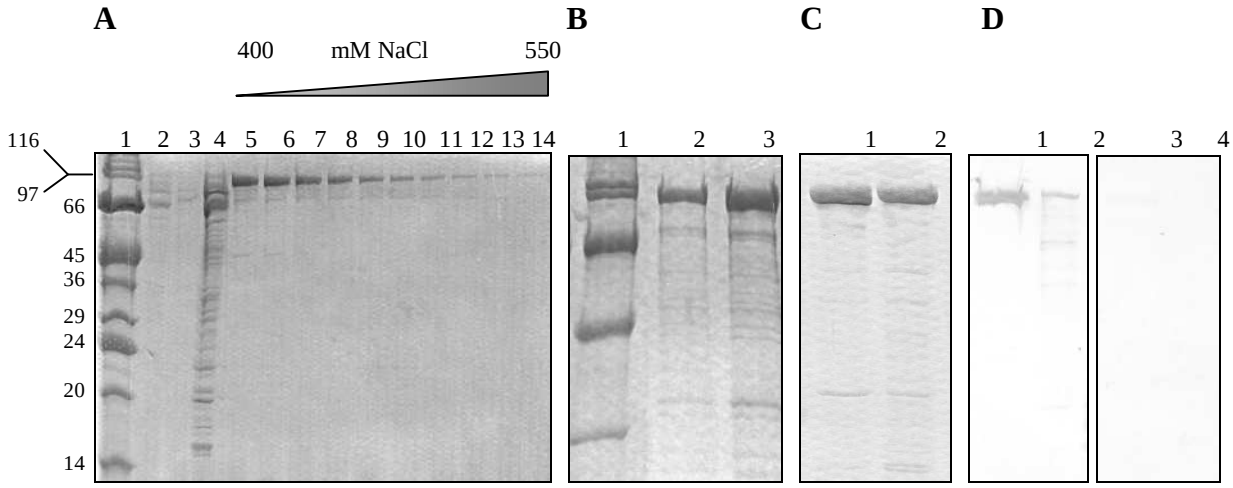


Figure 11. Purification of overexpressed protein Hsp93. A. After solubilization in 8 M urea, Hsp93 was purified using anion-exchange ResourceQ column. The sample was washed with 50 mM NaCl in Tris/HCl pH 8. 1/10 of the wash fractions was loaded on the gel (lanes 2-4). Elution was performed using the gradient ranging from 50 mM to 700 mM NaCl in Tris/HCl pH 8. 1/10 of the fractions corresponding to the peak region (400-550 mM NaCl) were analyzed by SDS-PAGE (lanes 5-14). Lane 1 indicates a mixture of high and low molecular weight marker, 10 µl each. **B.** Concentrated sample after purification using ResourceQ column, 1 and 3 µl were analyzed by SDS-PAGE (lanes 2 and 3, respectively). Lane 1 indicates 10 µl of high molecular weight markers. **C.** After purification on MonoQ column samples were concentrated using Millipore membrane filters with the pore size of 30 kDa and prepared for antibody production in the final concentration of 1 mg/ml. 2 and 1 µl were analyzed by SDS-PAGE (lanes 1 and 2, respectively). **D. Testing of Hsp93 antibodies produced in rabbit.** 7.5 µl of chloroplast stroma (lanes 1 and 3) and 0.5 µg of purified Hsp93 protein (lanes 2 and 4) were loaded on SDS-PAGE and analyzed by western transfer and subsequent immunodecoration by α-Hsp93 antibodies (lanes 1, 2) or by preimmune serum (lanes 3, 4).

6.1.5 Immunoprecipitation of Tic110 by stromal chaperones

It has been observed that the native psTic110 associates reversibly with the Hsp93 and Cpn60 chaperones of the chloroplast stroma (Nielsen et al., 1997, Kessler and Blobel, 1996). It has been proposed that Tic110 together with Hsp93 and Tic40 most likely functions at a later stage of protein transport across the inner envelope, at the stromal side, by recruiting Hsp93, a proposed ATP-dependent import motor (Chou et al., 2003, Kikuchi et al., 2006). To investigate whether Tic110 interacts with those chaperones during its own import into chloroplasts, for aquiring the native conformation in the stroma, conditions for co-immunoprecipitation of soluble intermediates with stromal chaperones were established.

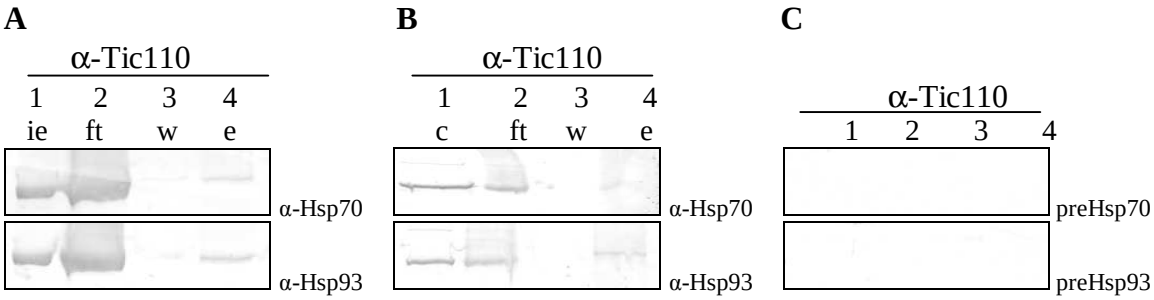


Figure 12. Co-immunoprecipitation of Tic110 by stromal chaperones Hsp93 and Hsp70. A. Inner envelope vesicles corresponding to 20 µg protein were solubilized and incubated with α-Hsp70 and α-Hsp93 antibodies for 90 minutes at RT. Antibodies were bound to ProteinA-Sepharose matrix during the subsequent 90 minutes incubation, rotating at RT. After washing and elution, samples were dissolved in Laemmli buffer and solubilized inner envelope (ie), 1/20 of the flow through after incubation with Protein A-Sepharose (ft), 1/10 from the last wash (w), and the elution (e) were analyzed by SDS-PAGE, western-blotted and immunodecorated with α-Tic110 antibodies. **B.** The same experiment performed using solubilized chloroplasts corresponding to 200 µg chlorophyll per reaction. **C.** In a control reaction with the preimmune serum against Hsp70, as well as Hsp93, chloroplasts (lane 1), inner envelope vesicles (lanes 2 and 3) and egg albumine (lane 4) were tested for interaction specificity and elution fractions analyzed by SDS-PAGE, western transfer and immunodecoration with α-Tic110 antibodies.

Intact chloroplasts or inner envelope vesicles were solubilized with 1.5% DeMa and immunoprecipitation using antibodies raised against stromal chaperones Hsp70 and Hsp93, α-Hsp70 and α-Hsp93, was performed (section 5.11). Antibodies against Hsp70, as well as Hsp93 interacted with Hsp70 and Hsp93 inside the inner envelope vesicles and co-immunoprecipitated Tic110 bound to those chaperones. The size of the bands shown corresponded to the size of Tic110 and was recognized by α-Tic110 antibodies (Figure 12A). This observation was confirmed by the analysis of solubilized chloroplasts (Figure 12B). Immunodecoration of preimmune serum control reaction with α-Tic110 antibodies did not show any signal, implying that the interactions between Tic110 and chaperones were specific.

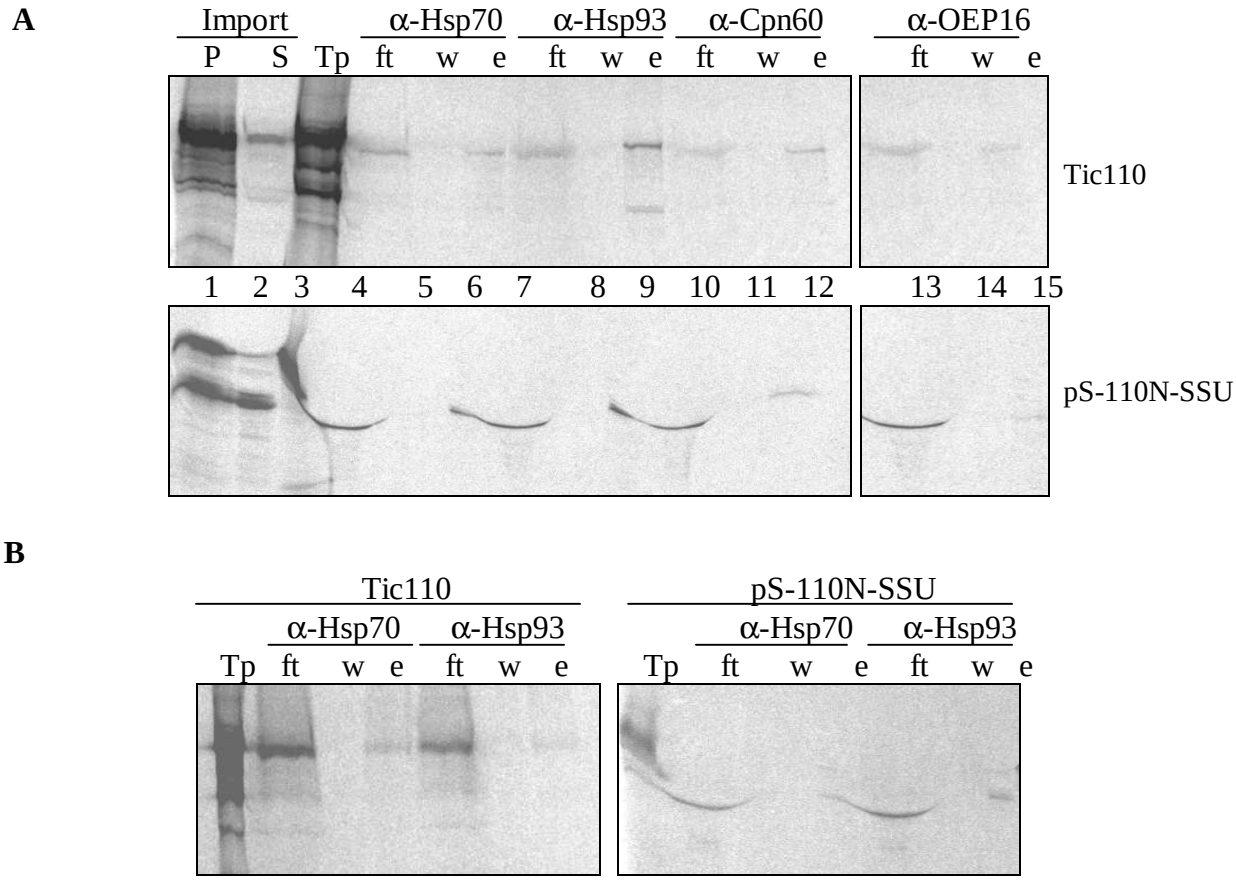


Figure 13. Co-immunoprecipitation of stromal intermediates of Tic110 and 110N-SSU by stromal chaperones. ^{35}S-pTic110 and ^{35}S-pS-110N-SSU were incubated with intact pea chloroplasts corresponding to 20 µg chlorophyll for 3.5 minutes at 25°C, in the presence of 3 mM ATP. After import, chloroplasts were reisolated on a Percoll cushion, washed and separated into the membrane and soluble fractions. **A.** The soluble stromal intermediates ^{35}S-Tic110 and ^{35}S-110N-SSU were dissolved in IP buffer and incubated with antibodies raised against stromal chaperones: α-Hsp70, α-Hsp93, α-Cpn60 and α-OEP16 as a control, 0.5 % egg albumine, and 0.3% DeMa for 1 hour at RT. Antibodies were collected using Protein A-Sepharose. The flow through after the incubation with Protein A-Sepharose (ft), the third wash (w) and the elution with Laemmli sample buffer (e) were analyzed by SDS-PAGE. The pellet and the supernatant after 3.5 minutes of import were loaded in lanes 1 and 2. Tp indicates 1/10 of the translation product used for each experiment. **B.** The membrane fractions of ^{35}S-Tic110 and ^{35}S-110N-SSU were solubilized in 1.5% DeMa and subsequently dissolved in IP buffer and treated as described in A. Incubation was performed with antibodies raised against stromal chaperones α-Hsp70 and α-Hsp93.

As described previously (Figure 8), the import time of 3.5 minutes has been selected for the purpose of the isolation of the soluble stromal intermediates of Tic110 and 110N-SSU. Radioactively labelled pTic110 and pS-110N-SSU were incubated with chloroplasts corresponding to 20 µg chlorophyll, in a standard import reaction containing 3 mM ATP, for 3.5 minutes at 25°C. After reisolation and washing steps, chloroplasts were separated into the pellet and soluble fractions. Soluble stromal import intermediates of Tic110 and 110N-SSU were subsequently incubated in immunoprecipitation buffer with antibodies raised against stromal chaperones Hsp70, Hsp93 and Cpn60. Antibodies with bound proteins interacted with

the Protein A-Sepharose, washed, eluted and analyzed by SDS-PAGE. Soluble Tic110 and 110N-SSU were both immunoprecipitated by all tested antibodies against stromal chaperones. Hsp93 interacted with Tic110 and 110N-SSU to the greatest extent, while the binding to the Hsp70 and Cpn60 was weaker. As a control, binding of the membrane fraction of Tic110 and 110N-SSU to stromal chaperones Hsp70 and Hsp93 was tested. The interaction of stromal chaperones and the mature Tic110 and 110N-SSU incorporated into the inner envelope membrane was very veak. This indicates that Tic110 interacts with stromal chaperones during its own import, before reinsertion into the inner envelope membrane. A slight binding to the control protein OEP16 was ascribed to the background unspecificity (Figure 13, lane 15).

6.2 Import properties of the chloroplastic intermembrane space proteins Tic22 and MGD1

To investigate import characteristics of the intermembrane space proteins Tic22 and MGD1, *in vitro* import experiments have been performed using ^{35}S-labelled precursor proteins, synthesized in the reticulocyte lysate system. It has been reported that Tic22 can be imported *in vitro* into pea chloroplasts, though at a very low rate (Kouranov et al., 1999). Tic22 contains a 6.3 kDa long chloroplast-targeting presequence that directs it to its final localization in the intermembrane space. Two homologues of Tic22 were found in *A. thaliana* genome: *At3g23710* and *At4g33350*. *At4g33350* is homologous to *P. sativum* psTic22 and has been used in this research. Import properties of MGDG synthases have not been investigated yet. Using the ChloroP neural network-based program prediction method (Emmanuelson et al., 1999) chloroplast transit peptides were predicted for the class A MGDG synthases (Figure 14). MGD1 from *A. thaliana* used in this research belongs to the class A of MGDG synthases and contains a predicted presequence of 106 amino acids (11.2 kDa). Alignments using the ClustalW program (Thompson et al., 1994) showed that the identity between the complete amino acid sequences of atMGD1 and cucumber or spinach MGDG synthases is around 70% and homology around 90%. By contrast, comparison of the class A MGDG synthases to the atMGD2 and atMGD3 shows much lower homology (around 70% homology and less than 50% identity).

Radioactively labelled precursors of Tic22 and MGD1 were incubated with intact chloroplasts in the presence of 3 mM ATP. This concentration of ATP is sufficient to allow

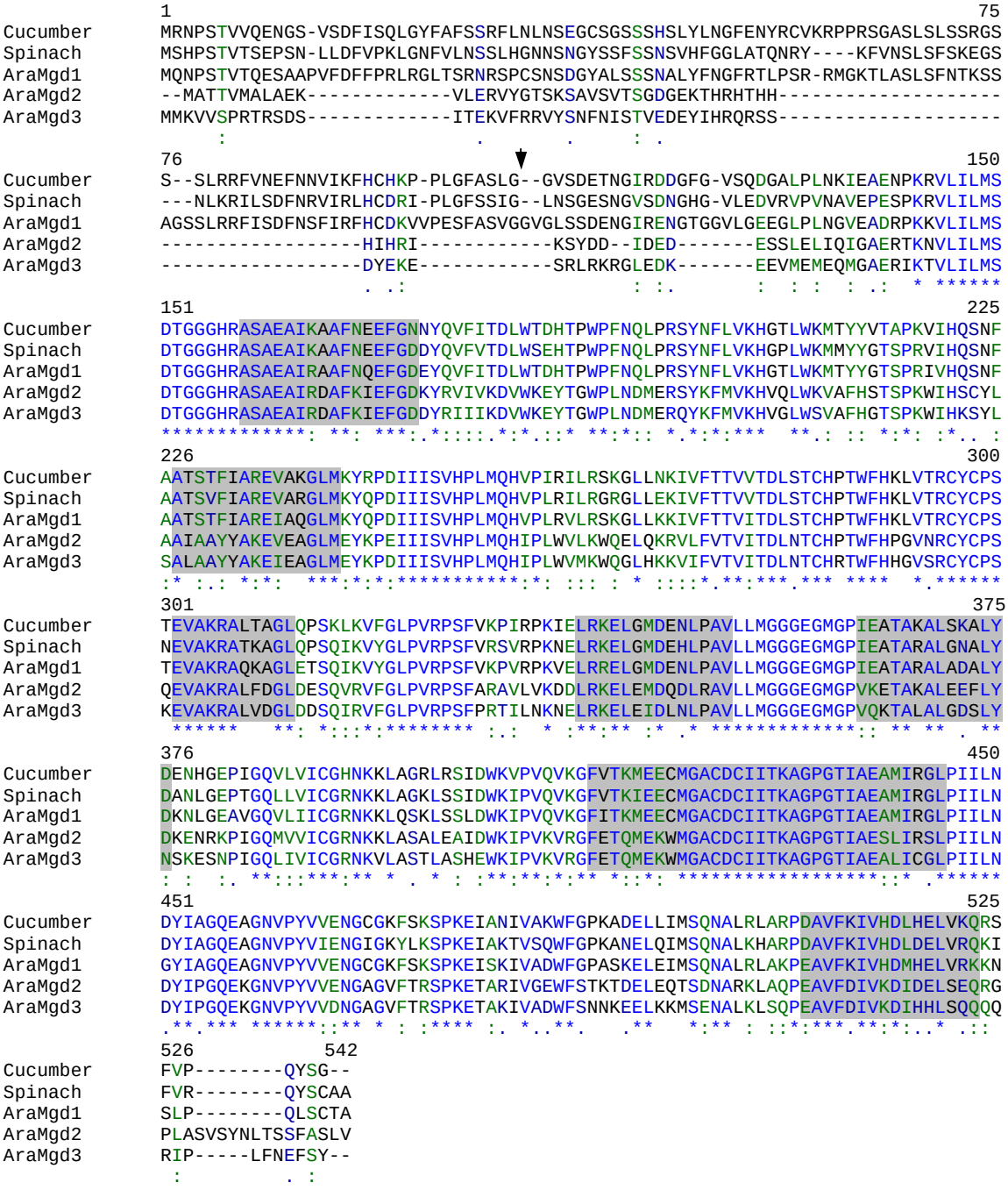


Figure 14. Comparison of amino acid sequences of cDNA encoding MGDG synthases from spinach, cucumber and *Arabidopsis*. The MGDG synthase sequences from *Arabidopsis thaliana*, atMGD1 (Ugt81A1), atMGD2 (Acc. No. AJ000331), atMGD3 (BAC F7E22); *Cucumis sativa*, csMGDA (Acc. No. U62622); and *Spinacia oleracea*, soMGDA (Acc. No. AJ249607) were compared with CLUSTAL W multiple sequence alignment program (Thompson et al., 1994). The transit peptide cleavage site for the cucumber MGDG synthase upstream of Gly 104 has been determined (Shimojima et al., 1997) and for atMGD1 upstream of Gly 107 was predicted as the cleavage site (Miège et al., 1999), indicated by the arrow. The amino acid numbers starting from the initiation methionine are indicated. Seven putative α-helices are boxed in gray. ***** - single, fully conserved residues; **:** - conservation of strong groups; **.** – conservation of weak groups.

complete translocation of proteins to any destination inside chloroplasts. After import chloroplasts were treated with thermolysin to remove proteins that were just bound to the chloroplasts surface. Thermolysin should not penetrate the outer envelope membrane in the concentration used for these experiments. Only the intermembrane space-, inner membrane-, and stroma-localized proteins remain protected after this treatment.

Only about 3% of pTic22 was processed to its mature form after import, visible as 22 kDa radioactive signal (mTic22), compared to the 28.5 kDa precursor protein (pTic22, Figure 15, lanes 1-3). After posttreatment of chloroplasts with thermolysin (0.5 µg per 1 µg chlorophyll), 50% of mature Tic22, as well as 50% of precursor form remained intact, protected from the protease action. On the other hand, treatment of the translation product with equivalent amounts of thermolysin led to the complete degradation of Tic22 (Figure 17A, lane 1). The experiments using a higher thermolysin concentrations revealed the same result, with around 50% protection from thermolysin treatment (compared to the import intensity without pretreatment). These results indicate that the observed forms are indeed imported into chloroplasts. A significant portion of a bound precursor of Tic22 that is protected from thermolysin digestion was probably translocated across the outer envelope membrane, but at this stage not yet processed to the mature form.

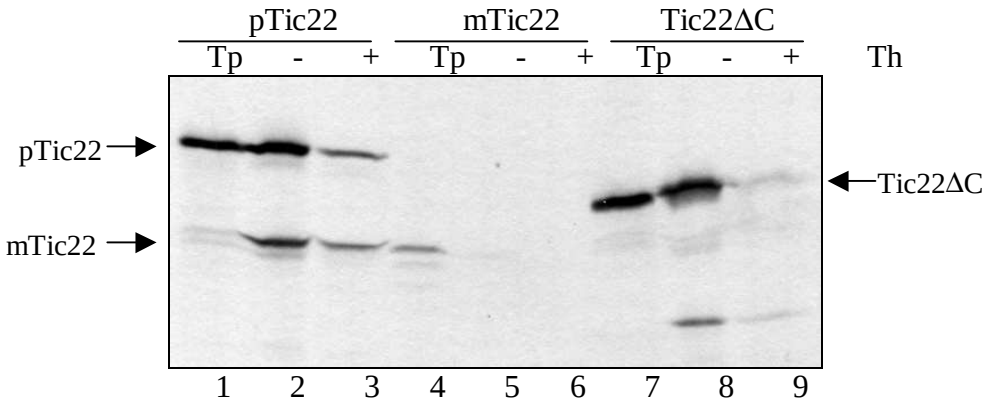


Figure 15. AtTic22 is imported into pea chloroplasts. *In vitro* synthesized ^{35}S-pTic22 (lanes 1-3), ^{35}S-mTic22 (lanes 4-6) and ^{35}S-Tic22ΔC (lanes 7-9) were incubated with isolated intact chloroplasts at 25°C for 20 minutes, in a standard import reaction containing 3 mM ATP. After import, samples were reisolated on a Percoll cushion and treated with thermolysin (lanes 3, 6 and 9). Results were analyzed by SDS-PAGE. Lanes 1, 4 and 7 represent 1/10 of the translation products used for the import reactions. The positions of pTic22, mTic22 and Tic22ΔC are indicated by arrows.

Import of the mature form of Tic22 could not be observed to any extent (Figure 15, lanes 4-6), indicating that the presequence is essential for successful targeting and translocation of Tic22 to the intermembrane space of chloroplasts. The construct lacking 44 C-terminal amino

acids was imported successfully into the intermembrane space (Figure 15, lanes 7-9). These results show that the C-terminus is not important for targeting and final positioning of Tic22, but instead, the complete targeting and localization information is located in its N-terminal targeting presequence.

Import of the radioactively labelled pMGD1 into intact pea chloroplasts resulted in the mature, thermolysin-protected product of around 45 kDa. Import was observed already without externally added ATP, but only after addition of 3 mM ATP the mature form was protected from thermolysin digestion (Figure 16A, lane 6 in comparison to lane 4).

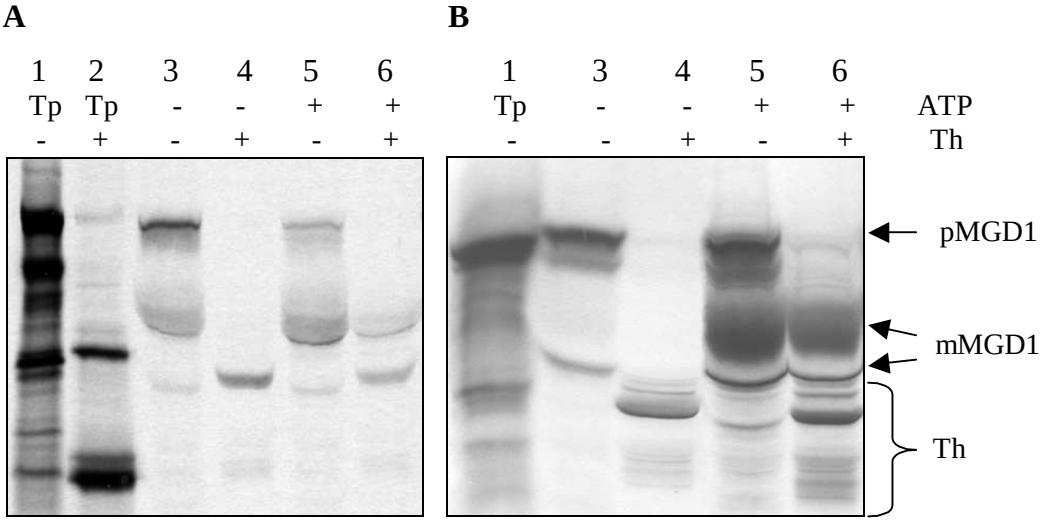


Figure 16. Import of atMGD1 into pea chloroplasts. A. *In vitro* synthesized ^{35}S-pMGD1 was incubated with isolated intact pea chloroplasts at 25°C for 20 minutes, in a standard import reaction. Lane 1 represents 1/10 of the the translation product used for the import. In lane 2 translation product was treated with thermolysin. Import was performed in the absence (lanes 3, 4) or presence (lanes 5, 6) of 3 mM ATP. After import chloroplasts were reisolated on a Percoll cushion and subjected to the treatment with 0.5 µg thermolysin per µg chlorophyll (lanes 4, 6). Untreated samples are shown in lanes 3 and 5. Results were analyzed by SDS-PAGE. **B.** Import of pMGD1 performed in the presence of 80 mM K_iPO_4. Precursor protein (pMGD1), mature form (mMGD1) and typical thermolysin degradation pattern (Th) are indicated.

Thermolysin digestion of the translation product revealed a typical pattern (indicated as Th in Figure 16) that was always present after the thermolysin treatment of chloroplasts after import, as well as after treatment of the translation product. Aiming to improve the low MGD1 import efficiency, the results from Hirsch and Soll (1995) were considered, since they showed that the import of an inner chloroplast envelope protein of 96 kDa is greatly stimulated by addition of potassium phosphate.

Though this method did not improve import of Tic22, it greatly stimulated import of MGD1. Addition of potassium phosphate buffer (80 mM, pH 7.6) increased the import efficiency of the previously observed 45 kDa form, as well as its other form that was running

slightly higher on SDS-PAGE (Figure 16B, lanes 5 and 6). The exact size of the mature forms of MGD1 was hard to determine because of its stretched and smeared shape. The observed bands most likely represent two forms of the same mature MGD1 and both show the same import behaviour. The lower, sharper and thinner, band represents a portion of mMGD1 that is pressed by the running behaviour of the large subunit of RubisCO and the upper, stretched and smeared, one was considered to indicate a portion of the mature MGD1 that is associated with lipids. 80 mM KPi buffer was used in all subsequent import experiments with MGD1.

Import of MGD1 into spinach chloroplasts was performed as well, because *Spinacea oleracea* as a 16:3 plant is expected to share more similarity with *Arabidopsis thaliana* concearning MGD1 import properties and topology than with the 18:3 plant *Pisum sativum*. Import into intact spinach chloroplasts, performed using the same conditions as for pea, gave the same result as shown in Figure 16. All subsequent import experiments have been performed in pea, because of the established conditions for pea growth and methods for intact chloroplasts and envelope isolation in our laboratory that could provide qualitatively and quantitatively uniform results.

6.2.1 Energy dependence of Tic22 and MGD1 import

Binding and spontaneous insertion into the chloroplast outer envelope membrane are the only steps of the protein import pathway that do not require energy in the form of ATP. For translocation of a protein across the outer envelope to the intermembrane space, or to the inner envelope and into the stroma, 50 µM to 1 mM ATP are required. The effect of an increase of externally added ATP on the import of Tic22 and MGD1 was tested in this study. Protein precursors were synthesized in reticulocyte lysate, a system that also contains an ATP regenerating system. This ATP can be depleted from translation products using gel filtration, apyrase treatment, hexokinase assay or combination thereof (as described in section 5.5.1). Intact pea plants were kept in the dark over night prior to chloroplast isolation and isolated chloroplasts were kept in the dark for at least 30 minutes prior to import. These incubations minimized the production of internal nucleoside triphosphates inside the chloroplasts by photophosphorylation, as well as carbohydrate metabolism. The import reaction was incubated in the dark for the same reason. Consequently, only the externally added ATP was supposed to influence the import rates of proteins tested. As a control for the energy state of chloroplasts during the import reaction, binding and import of pSSU was monitored.

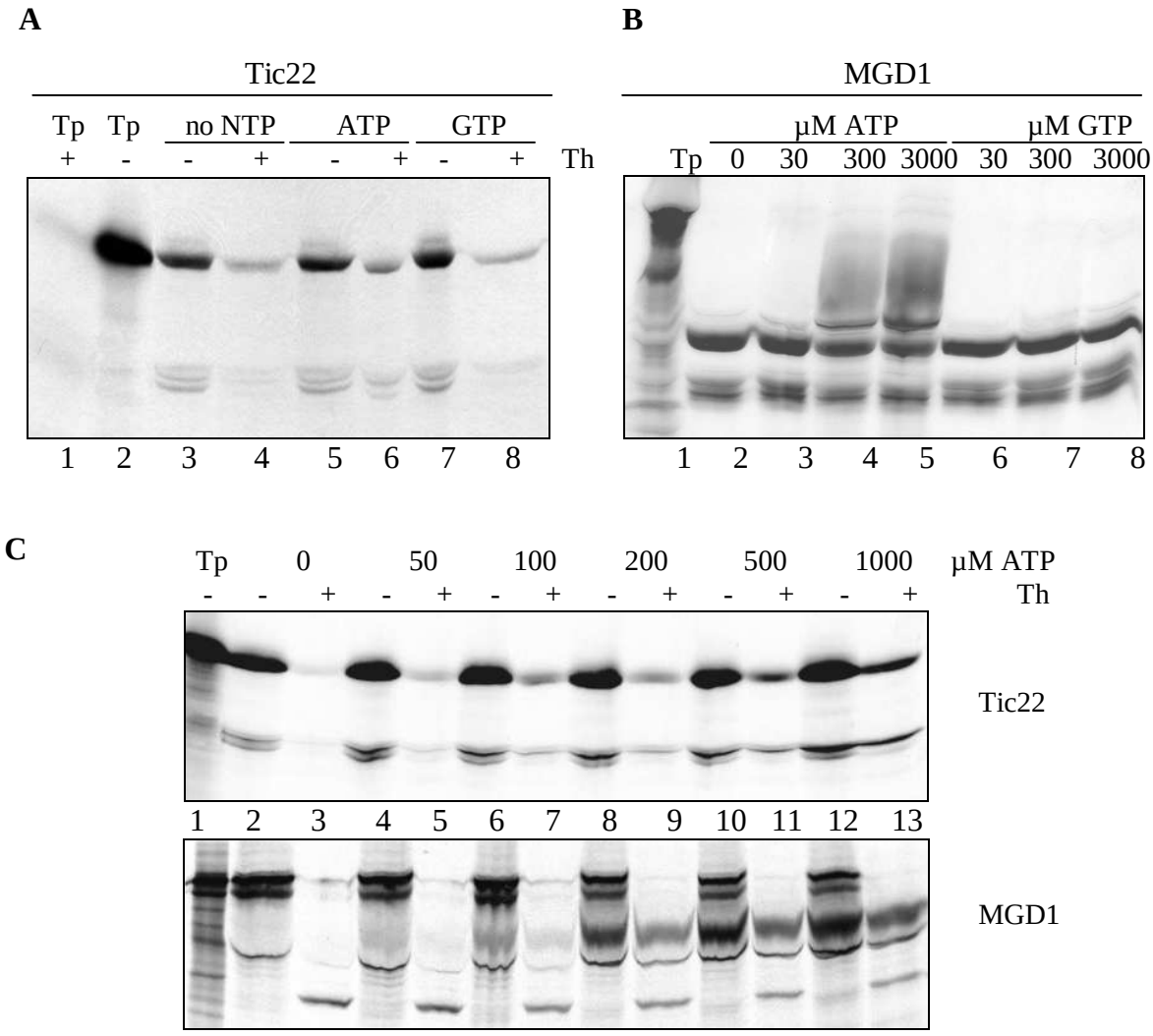


Figure 17. ATP-dependence of Tic22 and MGD1 import. Import into intact pea chloroplasts was performed by incubating *in vitro* synthesized ^{35}S-pTic22 or ^{35}S-pMGD1 with chloroplasts corresponding to 20 µg chlorophyll, at 25°C in a standard import reaction in the dark. Results were analyzed by SDS-PAGE. Tp represents 1/10 of the translation product used for import. **A.** The import of ^{35}S-pTic22 translation product was performed in the presence of 3 mM ATP (lanes 5 and 6), 3 mM GTP (lanes 7 and 8) or without NTP (lanes 3 and 4). Samples in lanes 1, 4, 6 and 8 were treated with thermolysin after import. **B.** ^{35}S-pMGD1 translation product was imported in the presence of increasing concentrations of ATP (lanes 3-5) or GTP (lanes 6-8). All samples were treated with thermolysin after the import reaction. **C.** For import, increasing concentrations of ATP, from 0 to 1000 µM (lanes 2-13) were used. After import chloroplasts were reisolated on a Percoll cushion and incubated in the presence (lanes 3, 5, 7, 9, 11, 13) or absence (lanes 2, 4, 6, 8, 10, 12) of 0.5 µg thermolysin per µg chlorophyll.

Radioactively labelled pTic22 and pMGD1 were imported into intact pea chloroplasts corresponding to 20 µg chlorophyll, for 20 minutes at 25°C. Both Tic22 and MGD1 imported into chloroplasts already at very low ATP concentrations, but the processed forms were almost completely degraded by thermolysin already at the early time points (Figure 17C, lanes 2-5). Import in the presence of GTP, as the only NTP added to the import reaction, was not observed for any of the proteins (Figure 17A, lanes 7-8 and B, lanes 6-8). Posttreatment

with thermolysin showed that a portion of the protease resistant mature form increases with increasing ATP concentrations. After import of Tic22 into pea chloroplasts, two bands of similar size could be distinguished. Since the lower one was removed by thermolysin treatment, only the upper one, which was changing its intensity in response to changed import conditions, was considered as the mature form of Tic22.

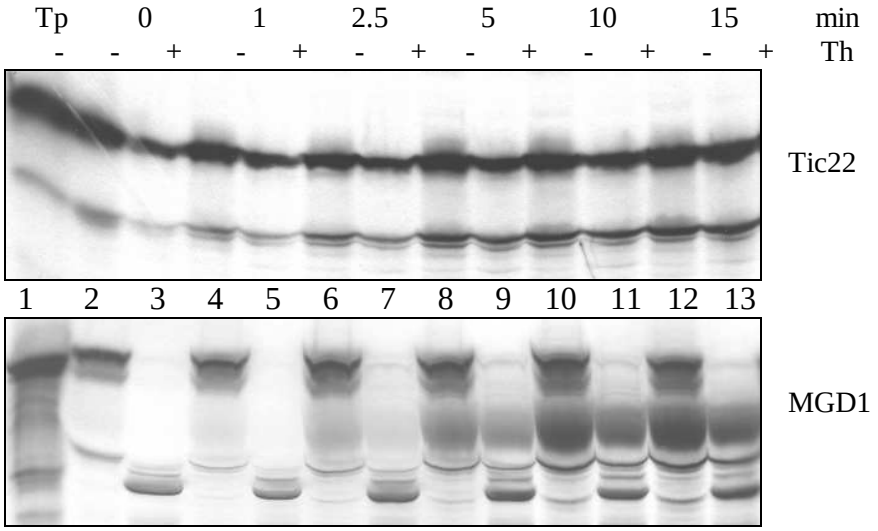


Figure 18. Time dependence of Tic22 and MGD1 import. Import into intact pea chloroplasts was performed using the standard protocol, by incubating *in vitro* synthesized ^{35}S-pTic22 and ^{35}S-pMGD1 with chloroplasts corresponding to 20 µg chlorophyll at 25°C in the presence of 3 mM ATP. The time of import reaction varied from 0 to 15 minutes (lanes 2-13). After import, chloroplasts were reisolated on a Percoll cushion and incubated in the presence (lanes 3, 5, 7, 9, 11, 13) or the absence (lanes 2, 4, 6, 8, 10, 12) of 0.5 µg thermolysin per µg chlorophyll. Results were analyzed by SDS-PAGE. Lane 1 represents 1/10 of the translation product used for import.

Both forms observed for MGD1 showed the same ATP dependent import behaviour. After addition of 100-200 µM ATP, 50% of import was accomplished and 500 µM ATP was required for achieving the maximal import rate (around 3% for Tic22 and up to 20% for MGD1). Import experiments performed for various times using a standard import reaction in the presence of 3 mM ATP showed similar behaviour for both proteins (Figure 18). Sensitivity to protease treatment decreased with time for both Tic22 and MGD1.

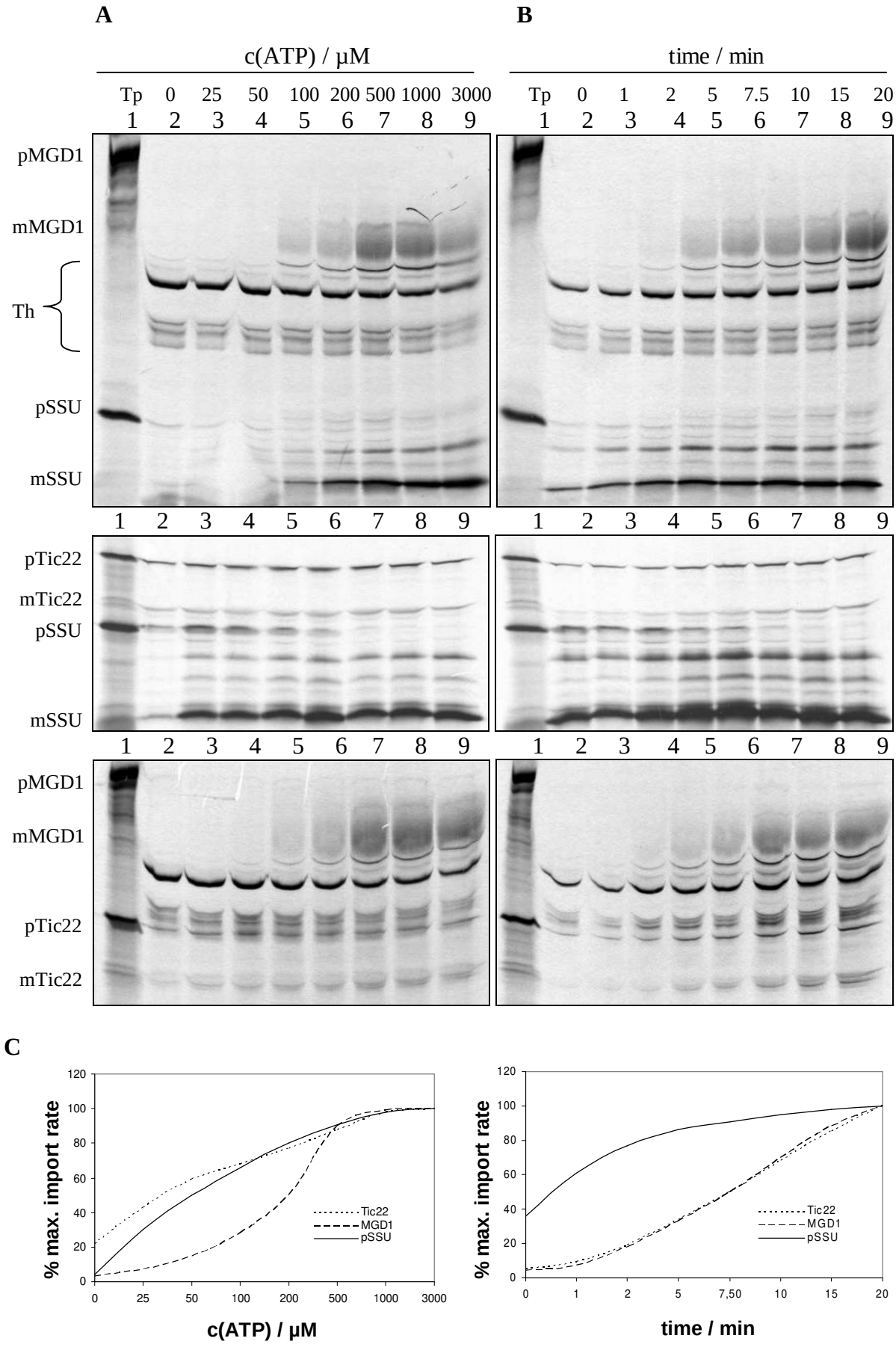

A
B
c(ATP) / µM
time / min
Tp 0 25 50 100 200 500 1000 3000
Tp 0 1 2 5 7.5 10 15 20
1 2 3 4 5 6 7 8 9
pMGD1
mMGD1
Th
pSSU
mSSU
pTic22
mTic22
C
% max. import rate
Tic22
MGD1
pSSU
c(ATP) / µM
time / min

Figure 19. Comparison of ATP- and time-demands for import of Tic22, MGD1 and SSU. Import into intact pea chloroplasts was performed using the standard protocol, by incubating *in vitro* synthesized ^{35}S-pTic22 and ^{35}S-pMGD1 with chloroplasts corresponding to 20 µg chlorophyll, at 25°C. Parallel imports combining ^{35}S-pMGD1 and ^{35}S-pSSU, ^{35}S-pTic22 and ^{35}S-pSSU and ^{35}S-pTic22 and ^{35}S-pMGD1, in the same reaction were performed. After import, chloroplasts were reisolated on a Percoll cushion and all samples were treated with thermolysin. Results were analyzed by SDS-PAGE. Lane 1 represents 1/10 of the translation product used for import. **A.** ATP-scale import into intact pea chloroplasts was performed using increasing concentrations of ATP from 0 to 3000 µM (lanes 2-9) for 15 minutes at 25°C. **B.** Time-scale import into intact pea chloroplasts was performed using increasing time from 0 to 20 minutes and 3 mM ATP at 25°C (lanes 2-9). **C.** ATP- and time-dependent import reactions from five independent experiments were quantified and results presented graphically.

The presence of high concentrations of ATP was sufficient for partial import of Tic22 even on ice (Figure 18, lanes 2 and 3), while MGD1 started to import efficiently only after around 2.5 minutes (Figure 18, lanes 6 and 7). After 10 minutes the import of both Tic22 and MGD1 was nearly completed (Figure 18, compare lanes 8 and 9 for Tic22 and 10 and 11 for MGD1).

To gain a better insight into the correlation between Tic22 and MGD1, imports under the influence of modification of ATP concentrations and time-scale were conducted, and to get a better comparison of import needs between those two intermembrane space proteins, parallel imports combining ^{35}S-pTic22 and ^{35}S-pMGD1, ^{35}S-pTic22 and ^{35}S-pSSU and ^{35}S-pMGD1 and ^{35}S-pSSU in the same reaction were performed (Figure 19). Results obtained by these experiments suggest that Tic22 imports into chloroplasts relatively quickly. Around 20% of total Tic22 imported, translocated without addition of external ATP (Figure 19A, lane 2) and the final maximal import rate was reached at 0.5 to 1 mM ATP (Figure 19A, lanes 7 and 8). The ATP-dependent import curve of Tic22 follows the one of the stromal protein pSSU, indicating that its import rate progresses in response to the increase of ATP concentration (Figure 19C). These results point to the conclusion that binding of Tic22 to chloroplasts does not require ATP. Also, a smaller portion of the preprotein is imported into chloroplasts under limited ATP conditions, but increase of the ATP concentration enhances its import and enables its complete translocation. In the presence of 3 mM ATP, already after 5 minutes at 25°C, 80% of maximal import rate for pSSU was reached (Figure 19B, lane 5, Figure 19C). In contrast, Tic22 and MGD1 require 10 to 15 minutes and 3 mM ATP to achieve the same import rate (Figure 19B, lanes 7 and 8, Figure 19C). More than 100 µM ATP was required for efficient import of MGD1, but its maximal import rate was achieved at 0.5 to 1 mM ATP, the same concentrations as observed for Tic22.

At this point it could be concluded that the import of MGD1 is dependent on and highly stimulated by ATP, while import of Tic22 is dependent on ATP hydrolysis to a lesser extent.

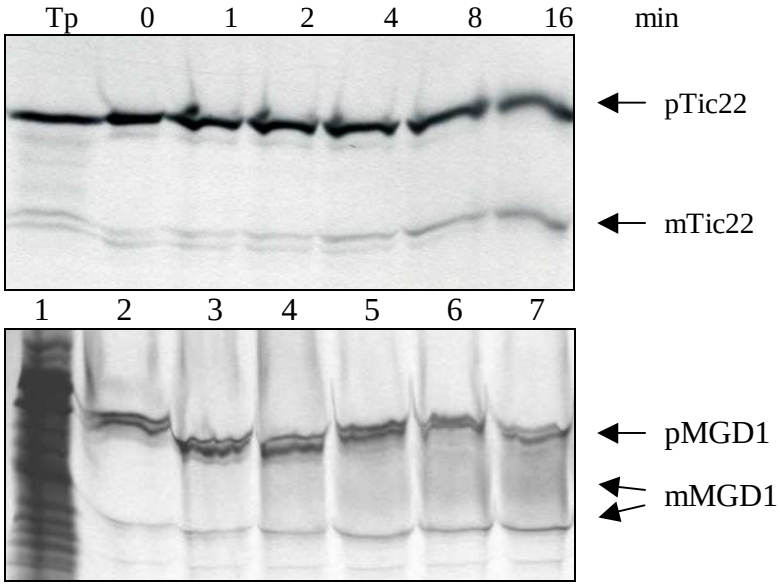


Figure 20. Pulse and chase of Tic22 and MGD1. Binding to intact pea chloroplasts was performed by incubating the *in vitro* synthesized ^{35}S-pTic22 and ^{35}S-pMGD1 with chloroplasts corresponding to 20 µg chlorophyll at 4°C in a standard import reaction lacking ATP. In the reaction mixtures containing MGD1 80 mM K_iPO_4 was added. After 2 minutes of binding, chloroplasts were reisolated and resuspended in the import mix containing 3 mM ATP, and import reaction was performed for different times at 25°C (lanes 3-7). Reaction was stopped by addition of Laemmli buffer and results were analyzed by SDS-PAGE. The control reaction was performed on ice (lane 2). Lane 1 indicates 1/10 of the translation product used for the import reaction.

Different needs for energy of those two proteins suggest that they might use different pathways for their import into chloroplasts.

Using a pulse-chase experiment (Figure 20), import behaviour of precursor proteins and arising of mature forms can be followed. Radioactively labelled precursors were incubated with intact pea chloroplast in a standard import reaction, using 20 µg chlorophyll. The reaction was incubated for two minutes on ice, without addition of ATP. Those conditions allowed binding of precursor proteins to the protein import receptors on the chloroplast surface. After 2 minutes the chloroplasts were pelleted and reisolated in a fresh import medium containing 3 mM ATP. The unbound precursors were removed by this step. After 1, 2, 4, 8, and 16 minutes import reactions were stopped by addition of Laemmli buffer and samples were analyzed by SDS-PAGE. After 4 minutes a significant increase in import rates of both Tic22 and MGD1 proteins could be observed (Figure 20, lane 5). The maximal import rate was achieved between 8 and 16 minutes (Figure 20, lanes 6 and 7).

6.2.2 Processing of the precursor proteins pTic22 and pMGD1

To address the question whether Tic22 and MGD1 use the same pathway for the translocation into chloroplast, determination of where the processing of precursor proteins takes place was attempted. The stromal processing peptidase is the only stroma-localized enzyme known to cleave signal peptides of preproteins in the course of the general import pathway (Abad et al., 1989, Richter and Lamppa, 1999). To investigate whether this enzyme is responsible for the processing of pTic22 and pMGD1, chloroplast stroma was isolated, and a stromal processing assay performed as described in the section 5.8. It was observed that pMGD1, as well as the control protein pSSU, was processed to its mature form in the presence of the stromal extract (Figure 21, indicated by arrows). After processing of pMGD1, a band of around 45 kDa could be observed (Figure 21, lanes 5 and 6). When chloroplasts were added to the sample to provide a lipid environment, the same pattern was observed as during the import of MGD1 (Figure 21B, lanes 3 and 4).

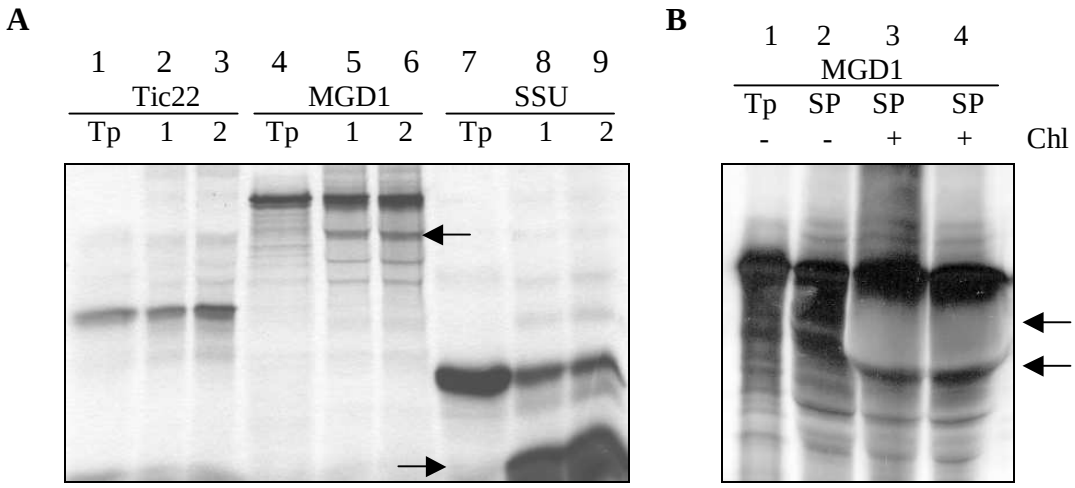


Figure 21. pMGD1, but not pTic22, is processed by the stromal processing peptidase. A. Stroma was isolated from pea chloroplasts and incubated with radioactively labelled translation products ^{35}S-pTic22, ^{35}S-pMGD1 and ^{35}S-pSSU for 90 minutes at 26°C. Reactions were stopped by addition of Laemmli buffer and samples were analyzed by SDS-PAGE. Lanes 1, 4, and 7 represent 2 µl of the corresponding translation products, and lanes 2-3, 5-6 and 8-9 represent 2 and 3 µl of the processed translation product. Mature forms of MGD1 and SSU, appearing after processing by a stromal processing assay, are indicated by arrows. **B.** After the stromal processing of ^{35}S-pMGD1, 5 µg of chloroplasts were added to the reaction, prior to the addition of Laemmli buffer. The lipid-associated form of MGD1 could be observed under those conditions.

The failure of the stromal extract to process the pTic22 (Figure 21A, lanes 2 and 3) led to the conclusion that Tic22 and MGD1 might use distinct pathways at a later stage of their import into the intermembrane space. In this scheme, MGD1 could transverse the Tic-machinery and

expose its N-terminal presequence to the stroma, where it is being cleaved and the mature protein is subsequently pulled back to its final location in the intermembrane space.

6.2.3 pTic22 and pMGD1 depend on proteinaceous components on the chloroplast surface for their import

Import of precursor proteins into chloroplasts requires protease-sensitive components on the outer envelope membrane. Thermolysin pretreatment can remove protein receptors on the chloroplast surface by proteolytic degradation. After this treatment, import of precursor proteins that require intact proteinaceous components should be inhibited. Intact chloroplasts were treated with 1 mg thermolysin per 1 mg chlorophyll for 30 minutes on ice in the dark. After treatment, intact chloroplasts were reisolated on a Percoll gradient in the presence of 5 mM EDTA and used for import reactions. In parallel, non-treated chloroplasts were used in control reactions. The radioactively labelled pTic22, pMGD1 and the control protein pSSU, which import is known to be dependent on thermolysin sensitive components on the chloroplast surface, were incubated with chloroplasts corresponding to 15 µg chlorophyll at 25°C for 10-12 minutes for pTic22 and pMGD1, and 5 minutes for pSSU. The experiments resulted in a significant effect of the thermolysin pretreatment on import of all tested proteins (Figure 22). The import yield of pSSU after thermolysin pretreatment was reduced to 30%, of pTic22 to 40% and of pMGD1 to 50% (Figure 22B and C), indicating that all these proteins need protein receptors on the chloroplast surface for the initial step of their import into chloroplasts.

6.2.4 pTic22 and pMGD1 compete with pOE33 for import into isolated pea chloroplasts

Import competition experiments were performed by binding of a single concentration of labelled translation product in the presence of various concentrations of unlabelled competitor protein. For the unlabelled competitor protein, like the oxygen-evolving complex precursor protein pOE33 used in this study, the import pathway is known. pOE33 uses the general import pathway through Toc- and Tic-complexes for its import into chloroplasts. Therefore, different concentrations of overexpressed and freshly purified pOE33 were added to a standard import reaction.

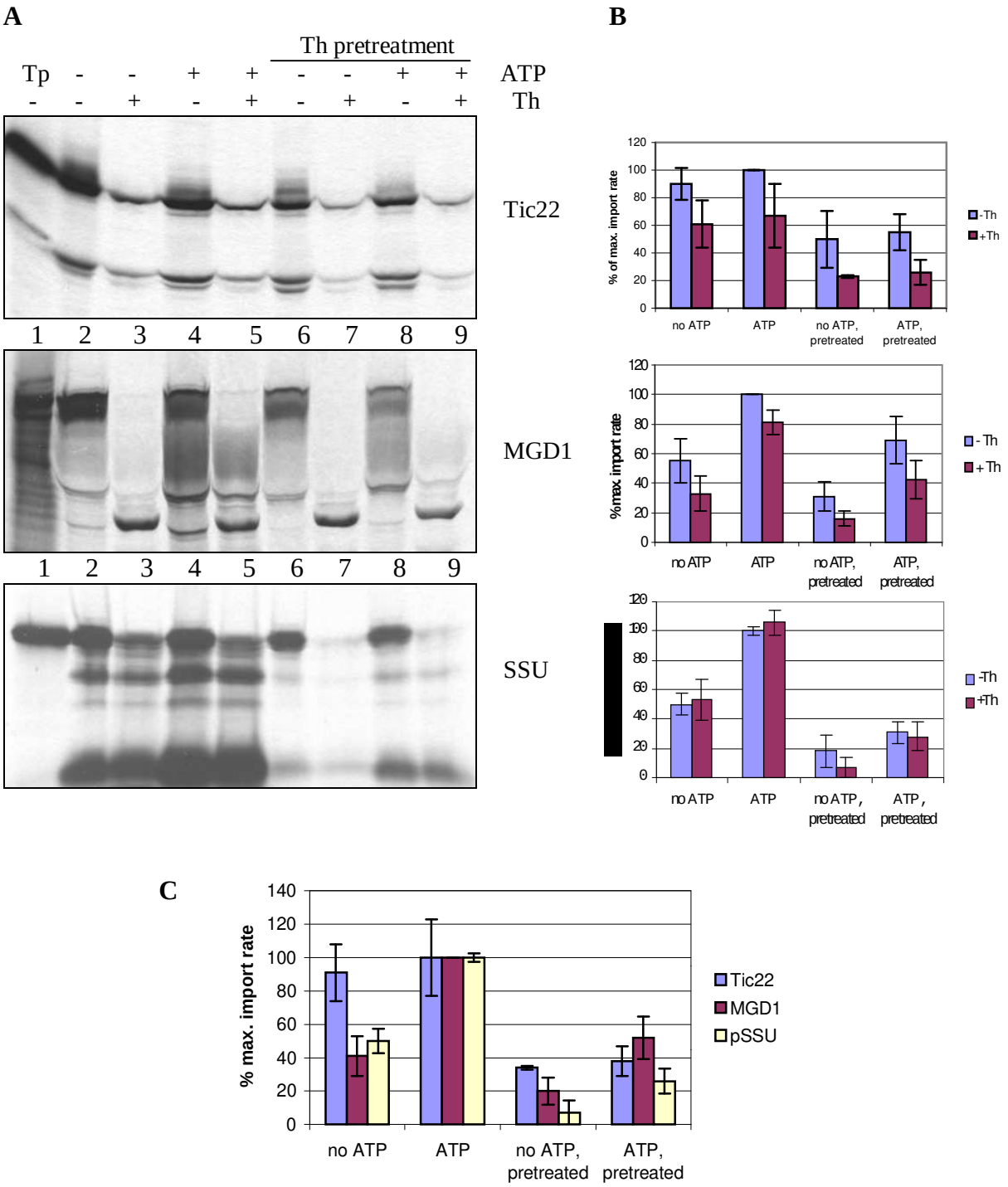


Figure 22. Import of pTic22 and pMGD1 is inhibited by thermolysin pretreatment of chloroplasts. A. For import, intact chloroplasts were used that were either pretreated (lanes 6-9) or not treated (lanes 2-5) with 1 mg thermolysin per 1 mg chlorophyll. Import of ^{35}S-pTic22, ^{35}S-pMGD1 and ^{35}S-pSSU into intact pea chloroplasts corresponding to 15 µg chlorophyll was performed for 15 minutes at 25°C for pTic22 and pMGD1, and 5 minutes for pSSU. After import chloroplasts were either subjected to thermolysin posttreatment (lanes 3, 5, 7, 9) or not (lanes 2, 4, 6, 8). Lane 1 represents 1/10 of the translation product used for import. **B.** Graphical presentation of the influence of thermolysin pretreatment of chloroplasts on import of pTic22, pMGD1 and pSSU, derived by 2D densitometry evaluation (AIDA image analyser) of five independently performed experiments for each protein. **C.** Direct comparison of the effect of thermolysin pretreatment on import of pTic22, pMGD1 and pSSU, based on the results presented in B.

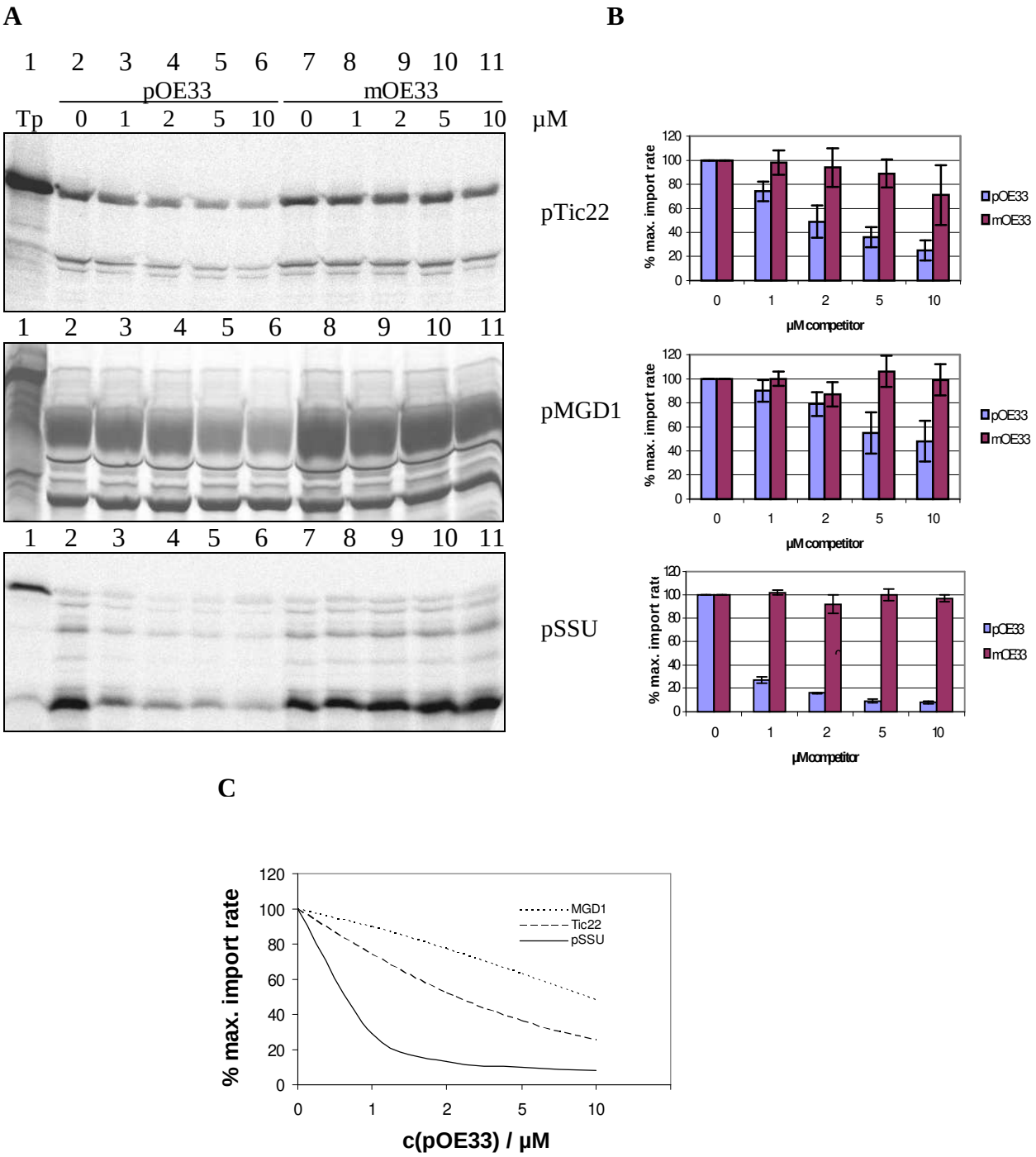


Figure 23. pTic22 and pMGD1 compete with pOE33 for import into chloroplasts. A. Increasing concentrations of overexpressed protein pOE33 (lanes 2-6) or its mature form mOE33 (lanes 7-10) were added into the import mix prior to import of ^{35}S-pTic22, ^{35}S-pMGD1 and ^{35}S-pSSU into intact pea chloroplasts corresponding to 15 µg chlorophyll. Import reaction was performed for 12 minutes at 25ºC for pTic22 and pMGD1 and 5 minutes for the pSSU control. After import, chloroplasts were subjected to thermolysin posttreatment. Lane 1 represents 1/10 of the translation product used for import. **B.** Graphical presentation of the results presented in A, estimated on the basis of five independently performed competition experiments. **C.** Direct comparison of pOE33 competition effect on import of pTic22, pMGD1 and pSSU, based on the results presented in B.

Radioactively labelled pTic22, pMGD1 and pSSU translation products were incubated in the presence of increasing concentrations of pOE33 (up to 10 µM). The import reaction lasted 10-12 minutes for pTic22 and pMGD1 and 5 minutes for pSSU.

Chloroplasts were added into the reaction mixture at the very end to avoid non-competitive import reactions of any of the proteins before all components of interest were added. Mature OE33, mOE33, was used as the negative control for this reaction. Because it lacks the presequence it cannot be recognized by the Toc-complex and cannot use the general import pathway. The results show that pOE33 exhibits the strongest effect on the import of pSSU, where application of just 1 µM pOE33 caused the decrease in import yield of about 75% (Figure 23). 2µM of pOE33 inhibited import of pTic22 to 50%, and after the application of 10 µM inhibitor only around 25% residual import remained. For pMGD1 this effect was slightly different. The maximal inhibition observed after application of 10 µM pOE33 was 50% (Figure 23). Control experiments using mOE33 as the competitor showed that it had no effect on the import of any of the tested preproteins. These results suggest that both pTic22 and pMGD1 competed for import with pOE33. Although the import of pMGD1 was competed to a lower extent than of pTic22, it is obvious that both proteins translocate into the intermembrane space through the Toc-translocon at the outer envelope membrane.

6.2.5 Toc34ΔTM competes with the endogenous Toc34 for binding to pTic22 and pMGD1 prior to import into isolated chloroplasts

Thermolysin pretreatment showed that pTic22 and pMGD1 require thermolysin sensitive components on the chloroplast surface for their import into chloroplasts. The results obtained by the competition experiments using pOE33 suggested that both proteins import into chloroplasts through the Toc-translocon at the outer envelope. Thus, it is probable that pTic22 and pMGD1 also use Toc34 as the initial receptor protein. The interaction of pTic22 and pMGD1 with Toc34 receptor GTPase was tested by performing binding experiments.

For the binding experiment Toc34 without the transmembrane domain (Toc34ΔTM) was overexpressed and purified under native conditions (section 5.10.4) and used fresh for further experiments. Purified Toc34ΔTM was preloaded with 3 mM GTP in the import mix, for 10 minutes on ice. Subsequently, ^{35}S-pTic22, ^{35}S-pMGD1 and ^{35}S-pSSU were added and incubation was prolonged for another 10 minutes on ice. After this time, chloroplasts corresponding to 15 µg chlorophyll were added and import was performed at 25°C for 10-12 minutes for pTic22 and pMGD1 and 5 minutes for pSSU.

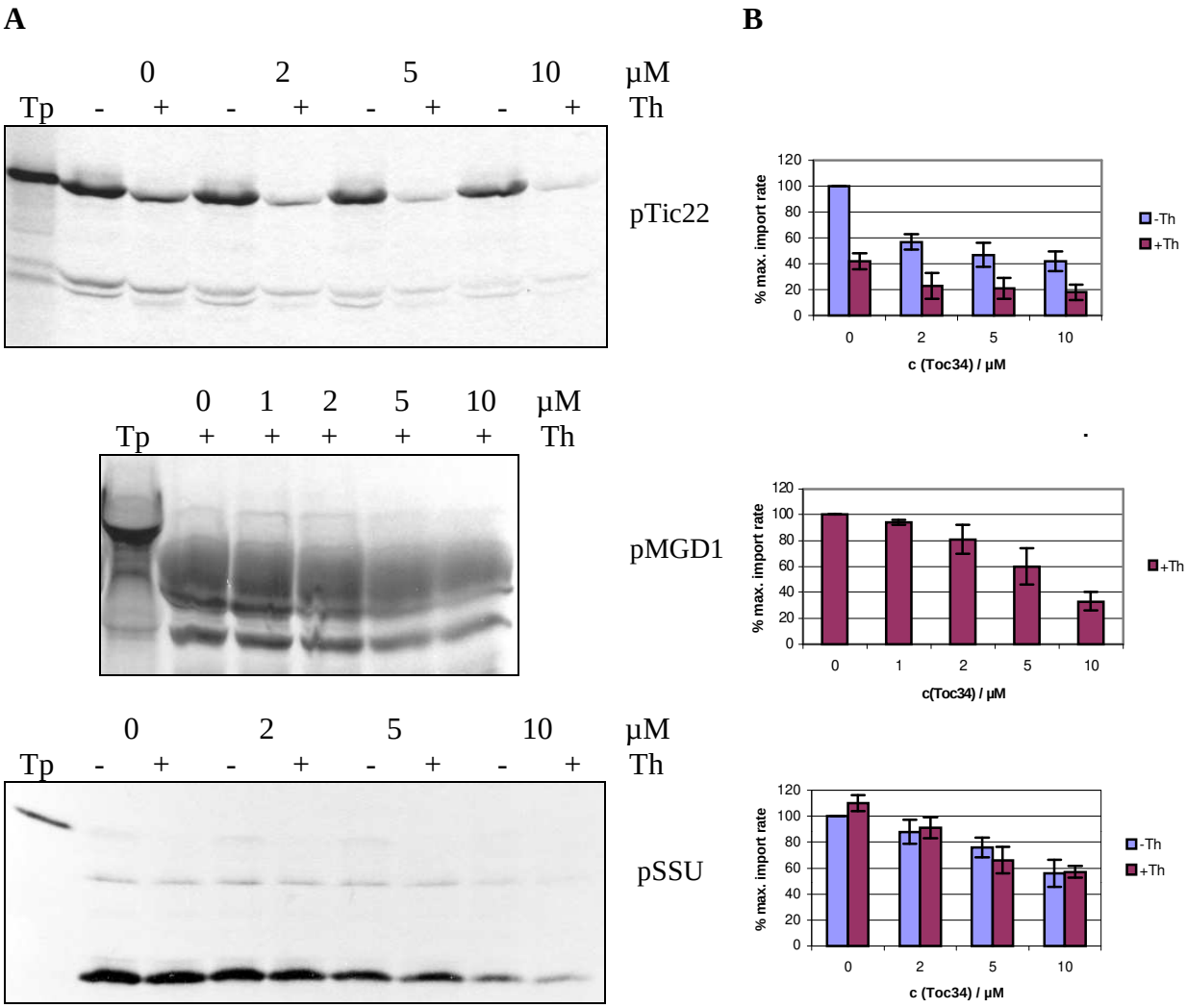


Figure 24. Import of pTic22 is inhibited by the soluble domain of the receptor protein Toc34. Increasing concentrations (0-10 µM) of overexpressed soluble receptor GTPase Toc34ΔTM, as well as 3 mM ATP and 3 mM GTP, were added to the import mix prior to import of ^{35}S-pTic22, ^{35}S-pMGD1 and ^{35}S-pSSU into intact pea chloroplasts corresponding to 15 µg chlorophyll. Import reaction was performed for 12 minutes at 25°C for pTic22 and pMGD1 and 5 minutes for the pSSU control. After import, chloroplasts were either subjected to thermolysin posttreatment (+Th) or not (-Th). Tp indicates 1/10 of the translation product used for import. **B.** Graphical presentation of the results presented in A, estimated on the basis of five independently performed competition experiments for each preprotein.

With increasing concentrations of Toc34ΔTM a decrease in import rate was observed for all tested proteins (Figure 24). This finding indicates that the soluble Toc34ΔTM interacts with precursor proteins prior to import and less of "free" precursor proteins remain to be imported into chloroplasts. The effect observed was the strongest for pTic22, indicating its strong interaction with Toc34. Interaction of MGD1 and Toc34ΔTM could be compared to the interaction with pSSU (Figure 24B), although the highest competitor concentration used (10 µM) caused stronger inhibition effect on import of MGD1 (~35% residual import compared to ~60% for pSSU).

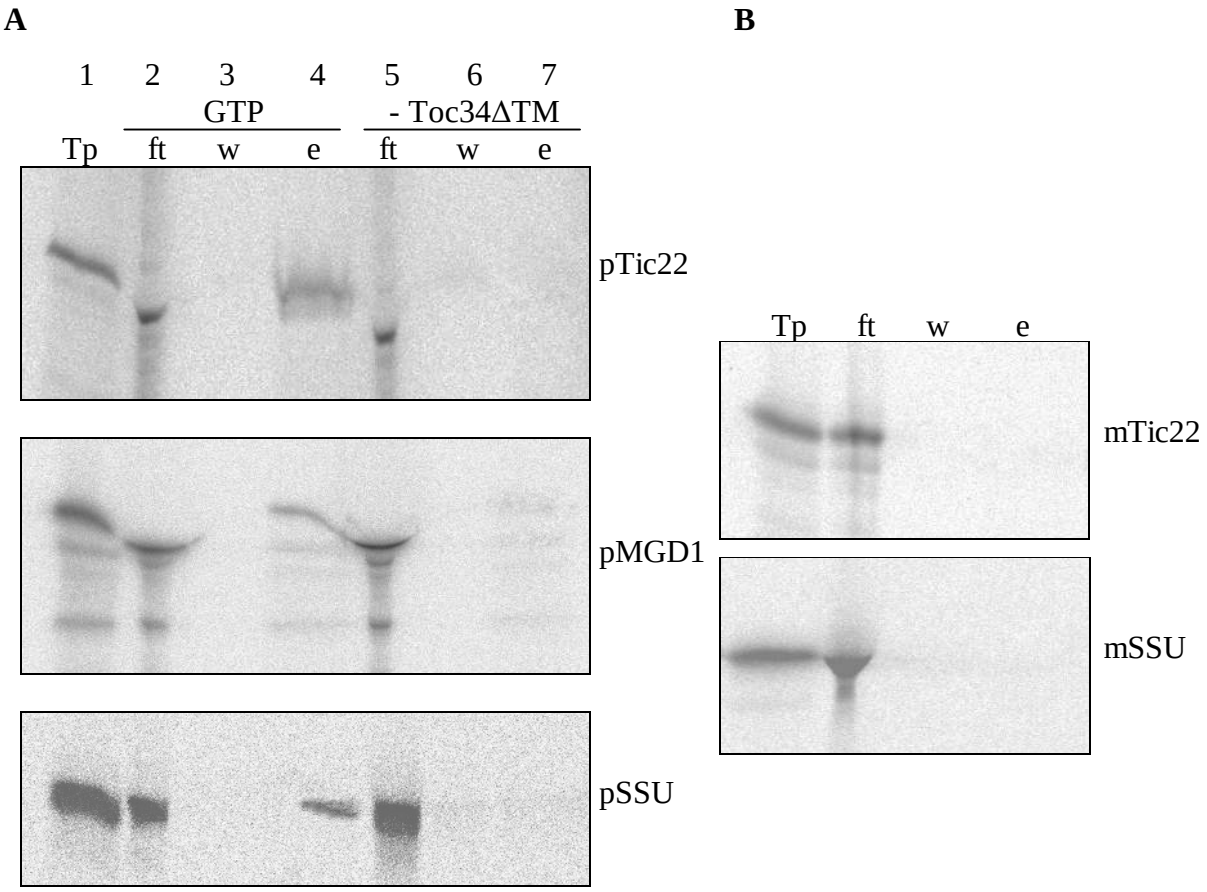


Figure 25. pTic22 and pMGD1 interact with the soluble domain of the receptor protein Toc34. A. For each separate experiment 300 µM of overexpressed soluble receptor Toc34ΔTM was coupled to 10 µl Ni-NTA matrix and preloaded with 1 mM GTP. Ni-NTA matrix without bound Toc34ΔTM was used as the negative control. ^{35}S-pTic22, ^{35}S-pMGD1 and ^{35}S-pSSU were added to the column in binding buffer and incubated for 45 minutes. The flow through after incubation (ft), the third wash of the matrix (w) and the elution with 250 mM imidazole (e) were analyzed by SDS-PAGE. Tp represents 1/10 of the translation product used in each experiment. **B.** Control experiment using ^{35}S-mTic22 and ^{35}S-mSSU in the previously described reaction.

To confirm the observed results another approach to test the interaction between the preproteins and the receptor GTPase Toc34ΔTM has been used. Freshly overexpressed and purified Toc34ΔTM was coupled to Ni-NTA column by the interaction of Ni^{2+} and the His-tag on the C-terminus of the protein. The protein was preloaded with 1 mM GTP in the binding buffer (see section 5.7). Subsequently, radioactively labelled translation products of pTic22, pMGD1 or pSSU were applied to the column, incubated for 45 minutes, washed, and Toc34ΔTM with bound preproteins was eluted by 250 mM imidazole and analyzed by SDS-PAGE. As controls, Ni-NTA matrix without bound Toc34 ΔTM, as well as mature forms of tested proteins (lacking the presequence) were used. The Toc34 ΔTM binding experiment has shown that both pTic22 and pMGD1, as well as the control protein pSSU, bind specifically to the Toc34ΔTM GTPase (Figure 25A, lane 4). Binding to the empty Ni-NTA matrix was not observed (Figure 25A, lane7). Binding of mTic22 and mSSU to Toc34 ΔTM

was tested as a control, using the same experimental setup. In both cases no binding could be observed (Figure 25B), indicating that Toc34ΔTM binds specifically to the presequences. Obtained results indicate that both pTic22 and pMGD1 use Toc34 as the initial receptor on their way into chloroplasts.

6.3 MGD1 overexpression and antibody production

For the purpose of overexpression and *in vitro* transcription, translation and import experiments, the original clone from *Arabidopsis thaliana* in pUNI51 vector was recloned into pET21d and pSP65 vectors. pMGD1 in pET21d vector failed to overexpress in any of the tested cell types. Also, when the first codons were replaced by those preferred by *E.coli*, this modification still did not influence the overexpression result. For the purpose of successful overexpression, the N-terminal 321 bp of the presequence have been removed and MGD1-P clone of 45.3 kDa was created.

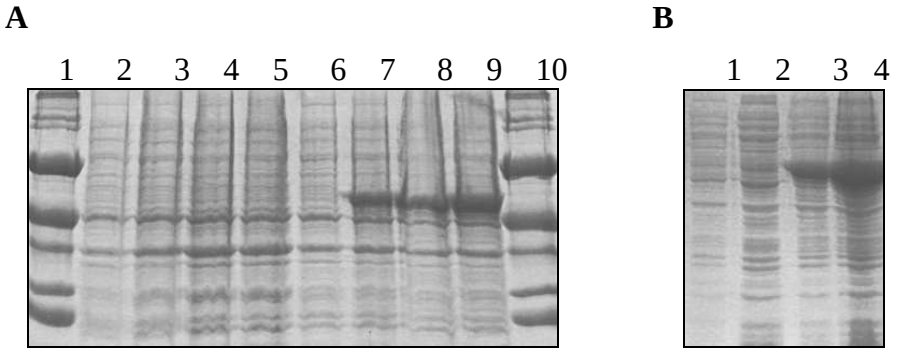
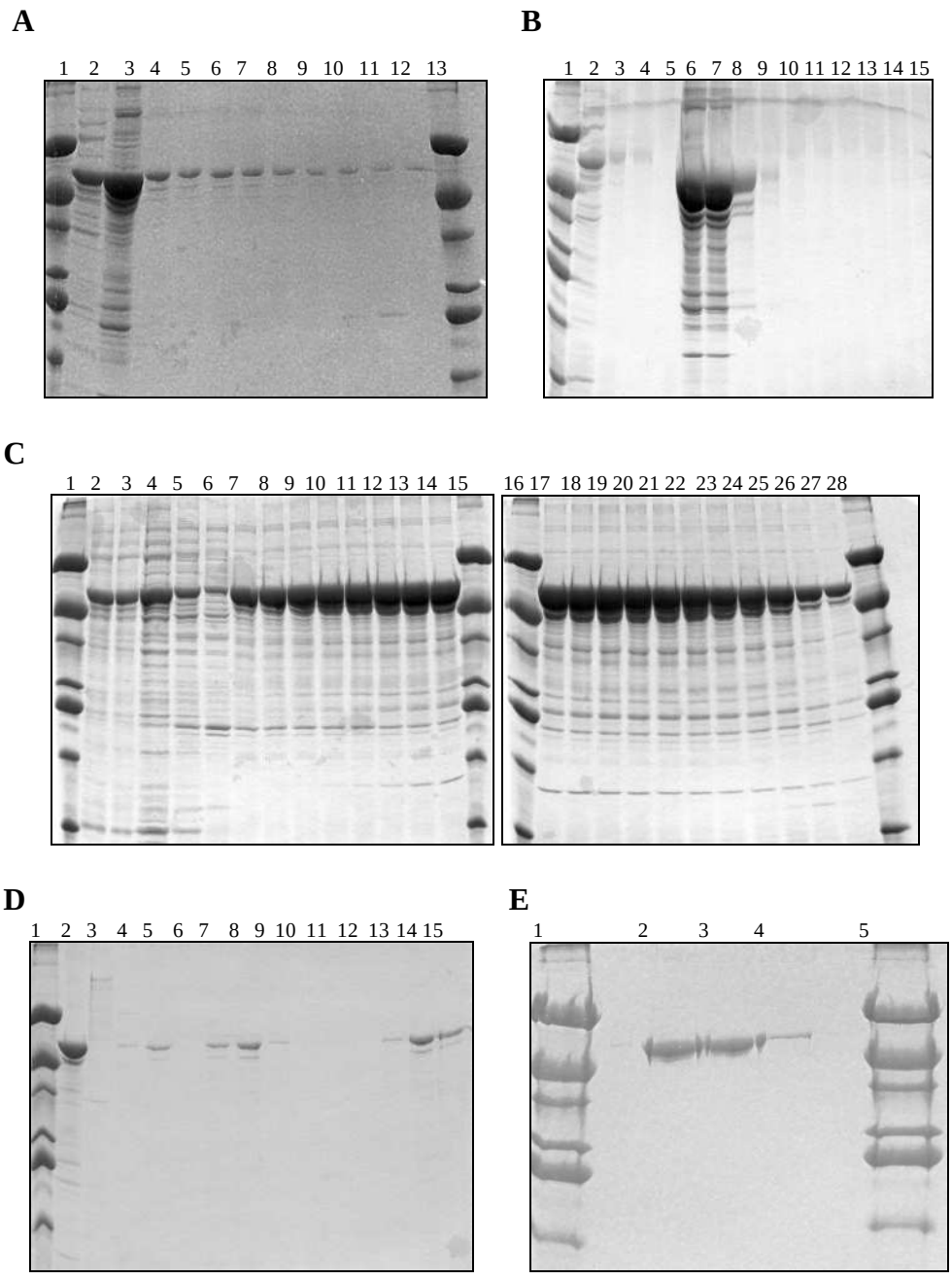


Figure 26. Overexpression of pMGD1 and MGD1-P clones. A. Overexpression of pMGD1 in pET21d vector (lanes 3-5) did not work. MGD1-P clone, lacking the presequence, has been constructed and successfully overexpressed in *E.coli* BL21(DE3) pRosetta cells (lanes 7-9). Lanes 2 and 6 represent control cells before induction with IPTG for pMGD1 and MGD1-P transformants, respectively. Lanes 3-5 and 7-9 represent samples taken after 1, 2 and 3 hours of overexpression. In lanes 1 and 10 is 15 µl of the low molecular weight marker. **B.** After overexpression, cells were separated into the soluble (lanes 1 and 2 contain 5 and 20 µl of sample) and in non-soluble fractions (lanes 3 and 4 contain 5 and 20 µl of the sample).

Overexpression was performed as described in section 5.10.2 and typical results are shown in the Figure 26. Overexpression of MGD1-P was successful and the protein was recovered as inclusion bodies. Further purification was performed under denaturing conditions, using 8 M urea. Because the protein could not be successfully purified on a Ni-NTA column using a pH-

or imidazole-gradient for elution (Figures 27A and B), or HiTrap column (Figure 27C), further purification using cation-exchange chromatography column PorousS was performed (Figure 27D). This method provided pure fractions of MGD1-P protein, in addition to an extra band, slightly smaller in size. This band was successfully removed by subsequently applied gel-elution technique (Figure 27E). A sample purified by this method was concentrated to 1 mg/ml and used for antibody production. Polyclonal antibodies were tested on purified protein MGD1-P and purified outer and inner envelope membranes from *Pisum sativum* (Figure 27F).



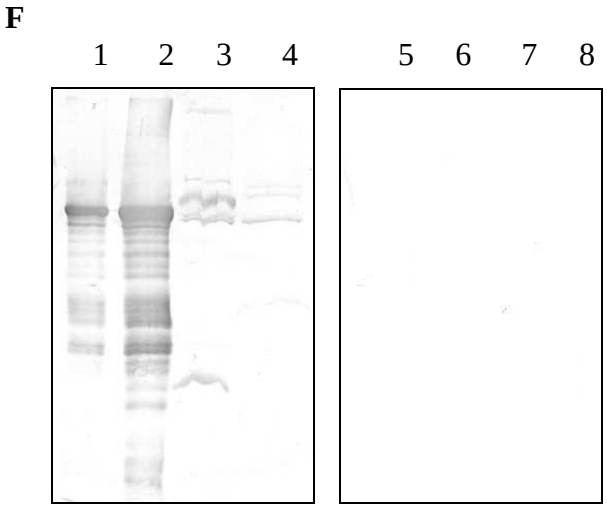


Figure 27: Purification of MGD1-P inclusion bodies. A. Purification on Ni-NTA column (His-tag), using a pH gradient elution. Lanes 1 and 13 represent 20 µl of a low molecular weight marker. In lane 2 is 1/1000 of the flow through, the column was washed with the buffer having a pH 6.3 (lanes 3 and 4), and eluted by the buffers of pH 5.9 (lanes 5-8) and pH 4.5 (lanes 9-12). **B.** Purification on a Ni-NTA column using imidazole gradient elution. Lane 1 represents 20 µl of a low molecular weight marker. In lane 2 is 1/1000 of the flow through, the column was washed with 10 mM imidazole (lanes 3-5) and eluted with increasing concentrations of imidazole (500 mM end concentration, lanes 6-15). **C.** Purification on HiTrap column (His-tag, Äkta) and elution by an imidazole gradient. The fractions under the peak area of the A_{280} absorption spectrum (higher protein amount) were tested. Lanes 2-3 represent the flow through, 4-6 the wash with 10 mM imidazole and 7-27 the elution with an imidazole gradient 20-500 mM. Lanes 1, 15, 16 and 28 indicate 20 µl of a low molecular weight marker. **D.** Purification by cation-exchange chromatography using a PorousS column. Lane 1 represents 20 µl of a low molecular weight marker. 20 µl of the sample before purification were loaded in lane 2. 30 µl of the flow through after applying the sample on the column is in lane 3. 1/10 of different fractions collected in the peak intervals are shown in lanes 4-15. **E.** The additional lower band was removed from MGD1-P by gel elution (lanes 2-4). Lanes 1 and 5 indicate 20 µl of low molecular weight marker. **F. Testing of MGD1-P antibodies produced in rabbit.** 0.65 and 1.3 µg of the purified protein (lanes 1-2 and 5-6) and 20 µl of the inner and outer envelopes (lanes 3-4 and 7-8) were analyzed by SDS-PAGE and western transfer, with subsequent immunodecoration with α-MGD1-P antibodies (lanes 1-4) or with preimmune serum (lanes 5-8).

6.4 Chemical crosslinking and immunoprecipitation

To investigate the other components of the Toc-translocon with which pTic22 and pMGD1 interact on their way inside the chloroplasts, chemical crosslinking and immunoprecipitation using antibodies against Toc34, Toc75, Tic110 and the outer envelope protein that is not a component of the import translocon, OEP16, were performed. Protein precursors ^{35}S-pTic22, ^{35}S-pMGD1 and ^{35}S-pSSU were bound to the receptors on the chloroplast surface after performing incubation in the import mix for 8 minutes on ice, or after inhibiting import by addition of $NiSO_4$ (see section 5.6.1.4). Chloroplasts were reisolated over a Percoll cushion and crosslinked using 0.5 mM DSP, as described in section 5.6.2.3. Crosslinked chloroplasts

were lysed, centrifuged, and resulting membranes solubilized in 1% SDS. Immunoprecipitation was performed by incubation of solubilized membranes with α-Toc34, α-Toc75(III), α-Tic110 and α-OEP16 antibodies (Figure 28A). Antibodies were collected using Protein A-Sepharose. The flow through after incubation with Protein A-Sepharose, the

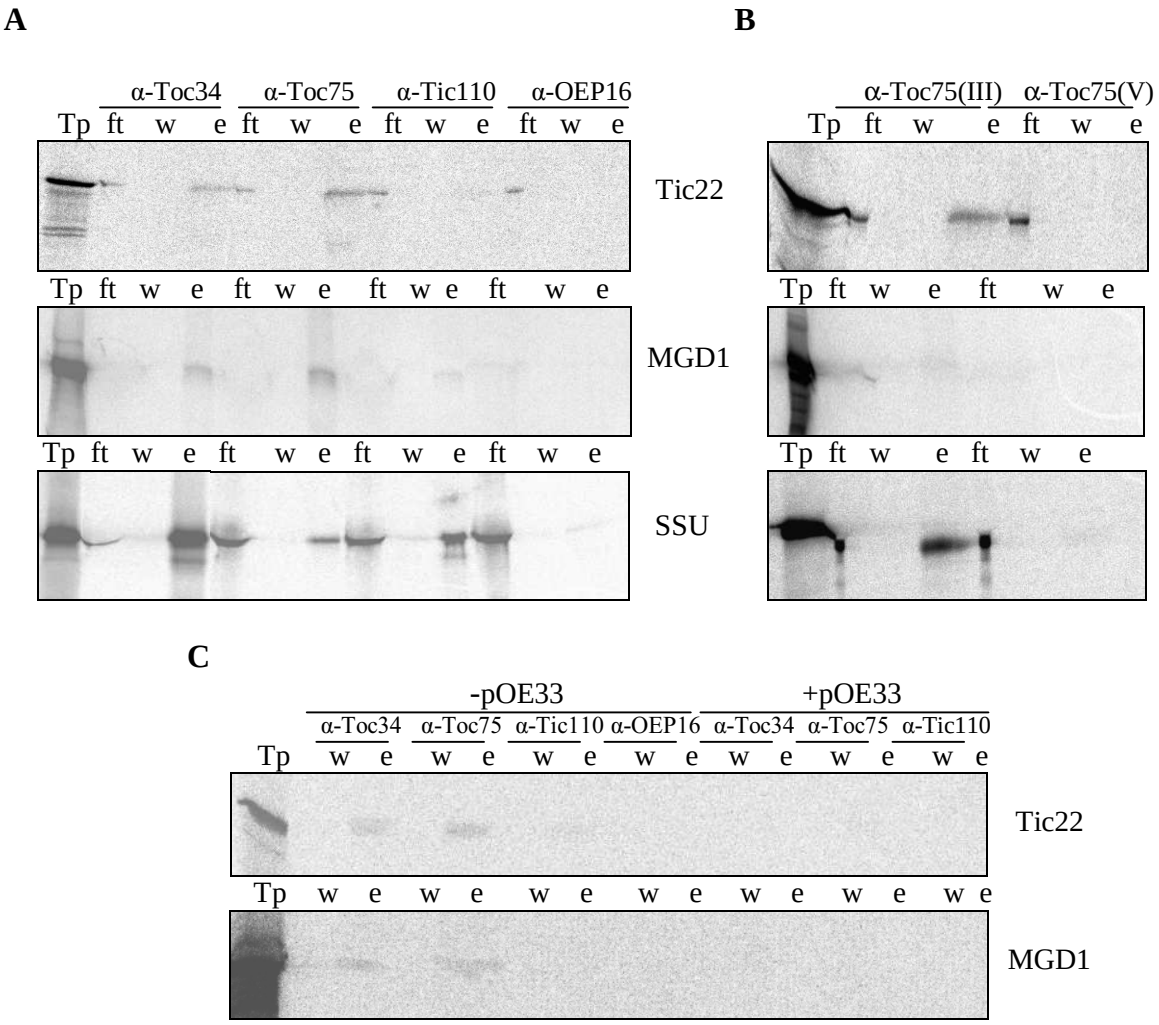


Figure 28. Chemical crosslinking and immunoprecipitation of pTic22 and pMGD1 to the major components of the translocation channel. A. ^{35}S-pTic22, ^{35}S-pMGD1 and ^{35}S-pSSU were incubated with intact pea chloroplasts corresponding to 20 µg chlorophyll for 8 minutes on ice. After reisolation on a Percoll cushion and subsequent washing, chloroplasts with bound precursor proteins were subjected to crosslinking using 0.5 mM DSP. The immunoprecipitation was performed after the lysis of chloroplasts, centrifugation and solubilization of the membranes. Antibodies raised against Toc34, Toc75, Tic110 and OEP16 were used for 1 hour incubation at RT. Antibodies were collected using Protein A-Sepharose. 1/10 of the flow through after incubation with Protein A-Sepharose (ft), 1/10 of the third wash (w), and the elution with Laemmli sample buffer (e) were analyzed by SDS-PAGE. Tp indicates 1/10 of the translation product used for each experiment. **B.** Crosslinking and immunoprecipitation were performed under the same conditions, using antibodies against two Toc75 isoforms: Toc75(III) and Toc75(V). **C.** Crosslinking and immunoprecipitation were performed in the absence or presence of 10 µM pOE33 in the import mixture.

third wash, and the elution with Laemmli sample buffer were analyzed by SDS-PAGE. pTic22 and pMGD1 were crosslinked to Toc34, Toc75 and to a lesser extent to Tic110 (Figure 28A).

It has been proposed that different isoforms of Toc and Tic components found in *Arabidopsis* join together to form different complexes with different preprotein specificity. It is believed that Toc33, Toc159, Toc75(III), Toc64(III), Tic22(IV), Tic110 and Tic40 join to form one type of the translocation complex, probably involved in the import of photosynthetic proteins; and on the other hand Toc34, Toc132 or Toc120, Toc75(III), Tic22(III) and Tic20(I) form the other type, probably involved in the import of the housekeeping genes (Vojta et al., 2004). Import through Toc75(V) has not been reported yet. To investigate whether Tic22 and MGD1 use the standard import pathway, comparison of crosslinking and immunoprecipitation between Toc75(III) and Toc75(V) isoforms have been tested (Figure 28B). Tic22 and MGD1 were found co-immunoprecipitated with Toc75(III) and not with Toc75(V). A control experiment using pSSU showed the same result. Also, crosslinking and immunoprecipitation were performed after binding of precursor proteins to the chloroplast surface in the presence or absence of 10 µM pOE33 (Figure 28C). The lack of a signal after chemical crosslinking and immunoprecipitation with antibodies raised against the components of the outer and inner envelope translocons proves that pOE33 in the import mix interfered with the binding of pTic22 and pMGD1 to the chloroplast surface. Acquired result indicates once again that all tested proteins use the same receptors at initial steps of their import. After binding to Toc34 (in pea) or Toc33 (in *Arabidopsis*), Tic22 and MGD1 are most likely transferred to the Toc159 and subsequently to the Toc75 translocation channel (Toc75(III)) on their way to the intermembrane space. From there Tic22 is probably released directly to the surface of the inner envelope, where it interacts with Tic110 and Tic20 (Kouranov et al., 1998), forming the Tic-translocon. MGD1 requires more energy for its import into the chloroplasts, as observed by performing ATP- and time-scale import experiments. MGD1 probably transfers through the Tic-translocon to the chloroplast stroma, where it is subsequently processed by the stromal processing peptidase. This translocation through the Tic-complex is most likely partial, because no soluble intermediates of MGD1 (nor of Tic22) have been observed by the experiments performed here. After processing, MGD1 has to reach its final destination in the intermembrane space.

6.5 Localization of MGD1

Tic22 is known to be located in the chloroplastic intermembrane space between the outer and the inner envelope membranes, and peripherally associated with the outer face of the inner membrane. For MGDG synthases from different plant organisms enzymatic activity has been investigated and it was proposed that the enzyme associates either with the outer envelope (cucumber, Shimojima et al., 1997), the inner envelope (*Arabidopsis*, spinach, Miège et al., 1999, Maréchal et al., 2000) or both envelope membranes (pea, Cline and Keegstra, 1983). Up to date, the question of the exact localization of the MGDG synthases has not been clarified. To resolve this problem, import of the *Arabidopsis* protein into chloroplasts isolated from *Arabidopsis*, or pea isoform into the pea chloroplasts had to be performed.

Import of MGD1 into intact *Arabidopsis* chloroplasts did not show interpretable results, and MGDG synthase from pea has not been characterized yet. For that reason import into pea chloroplasts was performed using the *Arabidopsis* protein atMGD1. Mature MGD1 was visible in two forms after import into pea chloroplasts (described in section 6.2). To investigate whether those two forms of MGD1 exist *in vivo* in *Arabidopsis* plants, the mature form of MGD1 imported into pea chloroplasts was compared with the one from mixed envelopes isolated from *A. thaliana*.

Import of MGD1 has been performed under the standard conditions, and after chloroplast reisolation and thermolysin treatment samples were solubilized in 6 M urea. The resulting samples were analyzed by SDS-PAGE and subjected to a western transfer and immunodecoration with α-MGD1-P (Figure 29A). At the end, the membrane was exposed on a film. The film (Figure 29B) and the membrane were overlayed after developing (Figure 29C). After comparing the samples treated with urea (Figure 29, lanes 2-11), it could be observed that the size and the shape of the mature form of MGD1 found in *Arabidopsis* envelopes correspond to the mature form of this protein determined by import into pea chloroplasts (compare lanes 10 and 8 and 9 in Figure 29C). Samples that were not treated with urea (Figure 29C, lanes 12-14) showed the same result: two distinct bands were visible in *Arabidopsis* envelopes, corresponding in size and shape to those derived by import into pea chloroplasts. These results indicate that the situation observed after *in vitro* imports of MGD1 into chloroplasts corresponds to the *in vivo* situation in *A. thaliana* and heterologous import of atMGD1 into pea chloroplasts gives rise to the mature product of the same size and shape as found attached to the *Arabidopsis* envelopes.

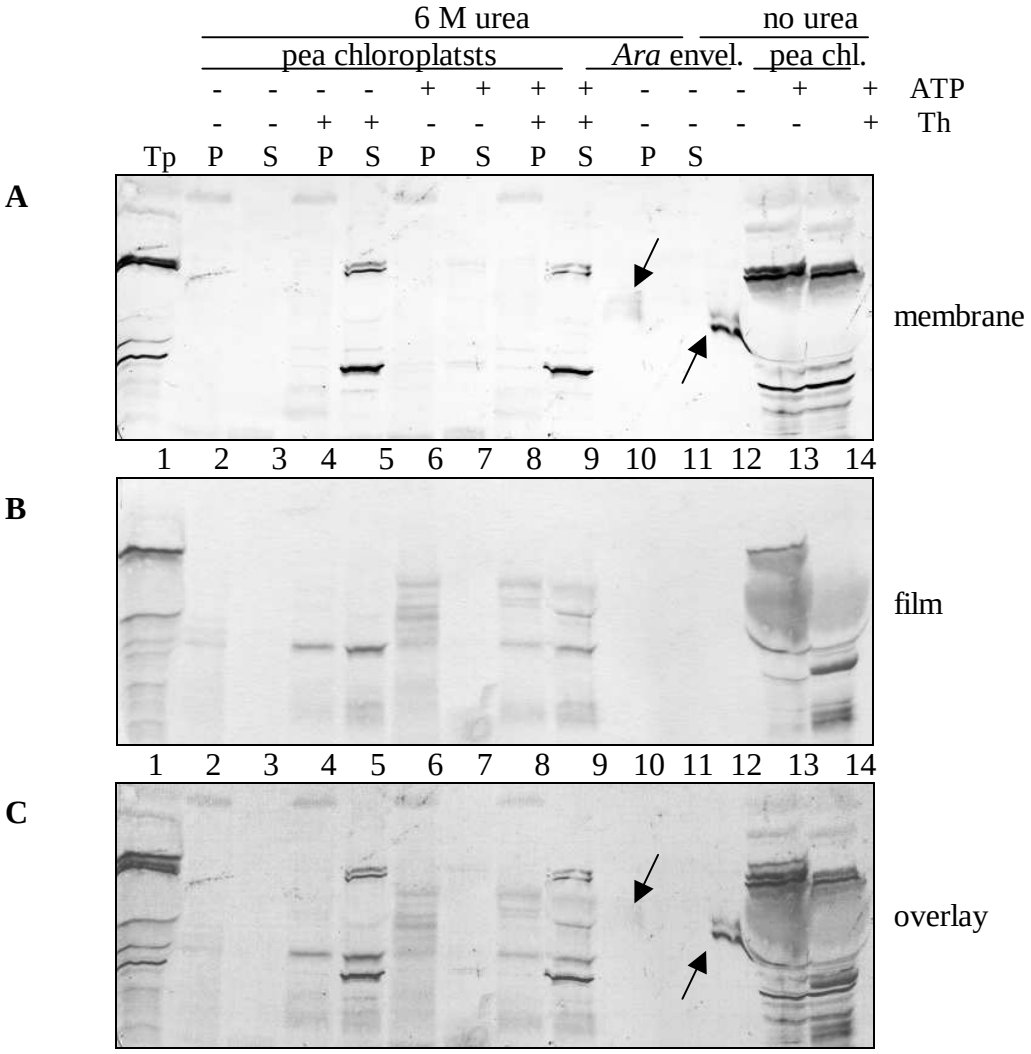


Figure 29. Size comparison of MGD1 between *Arabidopsis* envelopes and two forms visible after import into pea chloroplasts. ^{35}S-MGD1 was incubated with intact pea chloroplasts corresponding to 20 µg chlorophyll in a standard import reaction for 15 minutes at 25°C. The reaction was performed in the presence (lanes 6-9, 13 and 14) or the absence (lanes 2-5) of 3 mM ATP. After reisolation on a Percoll cushion and subsequent washing, some of the samples were subjected to the thermolysin treatment (lanes 4, 5, 8, 9, 14). Afterwards, chloroplasts were lysed and treated with 6 M urea for 20 minutes on RT (lanes 2-11). After centrifugation at 256,000xg for 10 minutes at 4°C, pellet (P) consisting of chloroplastic membranes and supernatant (S) were separated and analyzed by SDS-PAGE. A part of the sample was not treated by urea (lanes 12-14). Western transfer to a nitrocellulose membrane was performed, followed by immunodecoration using α-MGD1-P antibodies. Tp indicates 1/10 of the translation product used for the import reaction. **A.** Signals observed after the immunodecoration of the nitrocellulose membrane with α-MGD1-P antibody. **B.** Radioactive signals observed after exposure of immunodecorated nitrocellulose membrane on an x-ray film. **C.** Overlay of the x-ray film over the nitrocellulose membrane. Two forms of MGD1, observed in *Arabidopsis* envelopes, which correspond to the mature forms obtained by the import experiment, are indicated by the arrows.

Proteins that remain in the membrane fraction after treatment of membranes with 0.1 M Na_2CO_3 pH 11.5 or 6 M urea are probably integral membrane proteins. After treatment of MGD1 with 0.1 M Na_2CO_3, most of the precursor remained in the membrane fraction, which was specially clear in the samples imported under the presence of 3 mM ATP (Figure 30,

lanes 6-9). MGD1 degradation products derived from thermolysin posttreatment were found in the soluble fraction. Treatment of chloroplasts with 6 M urea resulted in the same effect. After the treatment, the protein was found in the membrane fraction (Figure 30, lanes 12-19), and only after thermolysin digestion a portion of the imported form was found to be soluble (Figure 30, lanes 15 and 19). The observed results were surprising, since MGD1 was predicted to be just associated to either the outer or the inner chloroplast envelope membrane. Only a weak association of MGDG synthases with envelope membranes by electrostatic interactions has been proposed (Shimojima et al., 1997, Maréchal et al., 2000). The results obtained here suggest that the interaction might be much stronger than speculated.

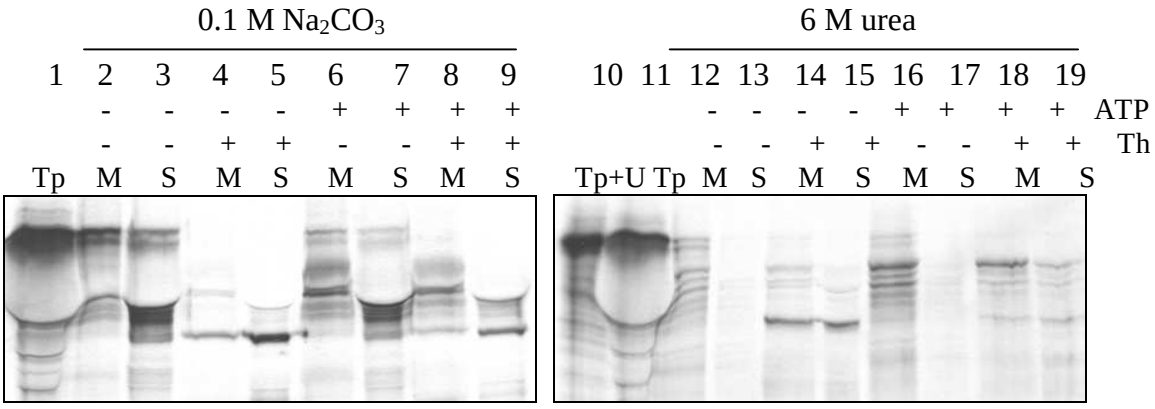


Figure 30. Extraction of MGD1 by 0.1 M Na_2CO_3 and 6 M urea, after import into chloroplasts. After import of ^{35}S-MGD1, chloroplasts were treated with 0.1 M Na_2CO_3 pH 11.5 (lanes 2-9) or 6 M urea (lanes 12-19) and separated into the membrane (M, lanes 2, 4, 6, 8, 12, 14, 16, 18) and soluble fractions (S, lanes 3, 5, 7, 9, 13, 15, 17, 19). Import was performed in the absence (lanes 2-5, 12-15) or presence (lanes 6-9, 16-19) of 3 mM ATP. After import chloroplasts were either treated with thermolysin (lanes 4, 5, 8, 9, 14, 15, 18, 19) or not (lanes 2, 3, 6, 7, 12, 13, 16, 17). Lanes 1 and 11 indicate 1/10 of the translation product used for the import and lane 10 the translation product treated with urea.

To test the extent of an association of MGDG synthase to the membrane, extraction of isolated chloroplast envelopes from pea was performed. 6 M urea, 0.1 M Na_2CO_3 pH 11.5 and 1 M NaCl were used to treat inner and outer envelope membranes corresponding to 20-30 µg protein for 20 minutes at RT (Figure 31). After the treatment, envelopes were centrifuged and the resulting pellet and supernatant were analyzed by SDS-PAGE and western transfer. α-MGD1-P antibodies were used for immunodecoration of the nitrocellulose membrane after western-transfer and α-Tic22, α-Tic110 and α-Toc75 were used as the controls for envelope purity and as the extraction control.

In *Pisum sativum*, most of the MGDG synthase was found associated to the outer envelope (this finding corresponds to the results from Cline and Keegstra (1983), who localized the activity of MGDG synthase from pea to the outer envelope membrane) (Figure 31, lanes 13 and 14). Treatment with 0.1 M Na_2CO_3 pH 11.5 and 1 M NaCl gave similar results using either of the envelopes (Figure 31, compare lanes 3-6 and 9-12). The only difference was a small amount of protein detected in the soluble phase after treatment of the outer envelope vesicles (Figure 31, lanes 10 and 12 compared to 4 and 6). The fact that MGDG synthase from pea was not extracted from a membrane even by 1M NaCl indicates that the interaction between this protein and the membrane is achieved by relatively strong electrostatic interactions. In contrast, treatment with 6 M urea, gave different results for the tested envelopes. The portion of the protein associated with the inner envelope membrane was not extracted by 6 M urea (Figure 31, lanes 1 and 2), and the portion associated to the outer envelope was found mostly in the soluble fraction (Figure 31, lanes 7 and 8).

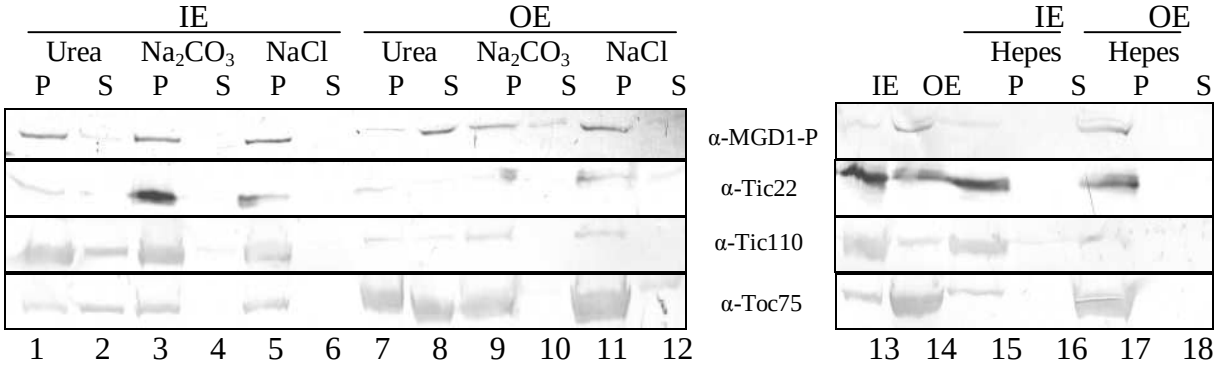


Figure 31. Extraction of MGDG synthase from inner and outer envelope vesicles from pea by 0.1 M Na_2CO_3, 6 M urea and 1M NaCl. Chloroplast envelopes were isolated as described in section 5.3. Afterwards they were pelleted at 256,000xg for 10 minutes at 4°C and resuspended in either 0.1 M Na_2CO_3 pH 11.5 (lanes 3, 4, 9, 10), 6 M urea (lanes 1, 2, 7, 8), 1 M NaCl (lanes 5, 6, 11, 12) for 20 minutes on RT, or in 10 mM Hepes/KOH pH 7.6 (lanes 15-18) for 20 minutes on ice, followed by centrifugation at 256,000xg for 10 minutes at 4°C. The pellet and the supernatant were analyzed by SDS-PAGE and western-blotting. α-MGD1-P, α-Tic22, α-Tic110 and α-Toc75 were used for immunodecoration of the nitrocellulose membrane after western-transfer. The distribution of the components between the two envelope membranes can be estimated by comparing lanes 13 and 14.

The experiment was repeated twice more using envelopes produced in different isolation runs, and the results were confirmed in both cases. These results show clearly that the MGDG synthase from pea associates with strong interactions to the inner envelope membrane, and just weakly attaches to the outer envelope membrane.

7 Discussion

The aim of this thesis was to investigate import properties of Tic110, Tic22 and MGD1 into isolated pea chloroplasts to define the pathway that each of those proteins uses for its translocation to the final destination: the inner envelope membrane for Tic110 or the intermembrane space for Tic22 and MGD1.

7.1 Import pathway of Tic110 into the inner envelope membrane of chloroplasts

By the mutual exchange of transit sequences between Tic110 and pSSU it has been shown that Tic110 utilizes the general chloroplast import pathway (Lübeck et al., 1997). But only when the hybrid protein pSSU-Tic110N-mSSU was used in import experiments, a soluble intermediate in the translocation pathway was clearly visible. The construct pSSU-Tic110N-mSSU was completely imported into chloroplast stroma, from which it could be recovered as a soluble, processed Tic110N-mSSU (Lübeck et al., 1997). Soluble Tic110N-mSSU was shown to enter a re-export pathway, which resulted in its insertion into the inner envelope membrane, and in the extrusion of large portions of the protein into the intermembrane space. Lübeck et al. (1997) have proposed that chloroplasts possess a protein re-export machinery for inner envelope proteins in which soluble stromal components interact with the membrane-localized translocation machinery. It was proposed that this translocation machinery could export a large polypeptide chain across the membrane into the intermembrane space. The components of the export machinery are unknown at the present time.

After processing of pSSU-Tic110N-mSSU in the stroma, Tic110N-mSSU is translocated to the inner envelope, as the mature form. From this it could be concluded that an export signal is not present in the transit sequence, but an internal targeting information is necessary. Since Tic110-mSSU successfully inserted into the inner envelope membrane it could be concluded that a re-export signal is localized in the N-proximal part of Tic110 (putative membrane anchor region), and is sufficient for targeting to the inner membrane and proper insertion. The soluble intermediate form of the full length Tic110 has not been observed in the stroma previously due to the low translation and import efficiencies of this protein (Lübeck et al., 1997). The translation efficiency of Tic110 has been improved in this

work by the addition of six methionines at the C-terminal end of the protein to enhance the intensity of the radioactive signal after translation. Tic110 followed the same import pathway, as observed previously for pSSU-Tic110N-mSSU. The processed form of Tic110 was found soluble in the stroma and, following how the import proceeds on a time scale, its re-export to the membrane was clearly visible. It was observed that just a portion of the protein (maximum 50% for Tic110N-mSSU and 15% for mTic110) was found soluble, while the larger portion of the mature protein was always recovered in the membrane fraction. From these experiments it could be proposed that a fraction of Tic110N-mSSU or mTic110 is never released from the membrane, indicating that the parallel mechanisms for the import into the inner envelope might exist: stop-transfer and conservative sorting. However, it seems unlikely that a protein would use two different import mechanisms *in vivo*. Since the soluble stromal intermediate of Tic110 has been persistantly observed, it is possible that the re-export process is highly efficient and that only very little soluble intermediate accumulates in the stroma. Therefore, it seems that Tic110 uses conservative sorting for its translocation into chloroplasts.

Recently, Li and Schnell (2006) reported that pTic40 from *A.thaliana* is imported into chloroplasts and processed to an intermediate size form (iTic40) before insertion into the inner membrane. iTic40 is soluble and inserts into the inner envelope membrane from the stromal compartment. Together with the results obtained in this work it can be proposed that inner membrane proteins are first imported into the stroma and subsequently inserted into the inner envelope in a re-export mechanism.

It has been observed that the native psTic110 associates reversibly with Cpn60 (Hsp60 homologue, Kessler and Blobel, 1996) and Hsp93 (chloroplast ClpC homologue, Akita et al., 1997, Nielsen et al., 1997) chaperones of the chloroplast stroma. Kessler and Blobel have proposed that Tic110 acts as a docking site for molecular chaperones at the inner membrane. Akita et al. observed that significant quantities of ClpC were immunoprecipitated with antibodies against Tic110 and proposed that ClpC fills the role played by mtHsp70 during protein translocation into mitochondria. Nielsen et al. first observed that the precursor, but not the mature form of SSU, was detected in the α-ClpC immunoprecipitated fractions. It has been proposed that the association of ClpC was specific to precursors associated with the chloroplastic protein import apparatus. Also, complexes that co-immunoprecipitated with ClpC contained Tic110, Toc159, Toc75 and Toc34, regardless of whether precursor was

present or not. Therefore, Nielsen et al. (1997) suggested that the interaction of Tic110 with ClpC and Cpn60 is not necessarily bound to the chloroplast protein import.

In this work, the soluble stromal intermediates of mTic110 and Tic110N-mSSU were reisolated after import and co-immunoprecipitation with stromal chaperones Hsp93 (ClpC), Hsp70 and Cpn60 was performed. Co-immunoprecipitation with all tested chaperones indicate that Tic110 interacts with stromal chaperones during its own maturation, and not only as a component of the translocation machinery. Co-immunoprecipitation with Hsp93 was of the highest intensity, indicating that this chaperone might play a major role in helping Tic110 to reach the native conformation, and Hsp70 and to lesser extent Cpn60 might play an accessory role in this process. Membrane-bound fractions of mTic110 and Tic110N-mSSU also precipitated together with Hsp70 and Hsp93, though at a very low extent. In this case binding to Hsp93 was very weak (Figure 13B), which contradicts the above mentioned results from Akita et al. (1997), performed *in vivo*.

7.2 Import pathway of intermembrane space protein Tic22

Arabidopsis Tic22 (*At4g33350*) shows high homology to Tic22 from *P. sativum* and therefore it has been expected to import into intact pea chloroplasts *in vitro*. Indeed, although at a very low yield (3%), atTic22 imported into isolated pea chloroplasts. Import of Tic22 is dependent on its N-terminal presequence (Figure 15), which is necessary and sufficient for targeting to the intermembrane space of chloroplasts. Import of Tic22 into chloroplasts required low amounts of externally added ATP. As a result of the protease treatment after import, both the precursor and mature forms of the protein remained protected from degradation to the same extent (mostly around 50%, Figure 15). It appears that, although imported, not the entire population of radioactively labeled precursor protein was successfully processed to the mature form. This effect has been observed previously (Kouranov et al., 1999). With increasing ATP concentrations more of the imported precursor and mature forms remain protected from protease digestion, indicating that although low amounts of ATP are enough for initial import of Tic22, 1 mM ATP is necessary to accomplish the maximal import rate (Figure 17C). The same has been observed by varying the time of import. A portion of Tic22 was imported already on ice, but only after 10-15 minutes in the presence of 3 mM ATP, a significant amount of imported protein remained protected from protease degradation (Figure 18). It was clearly shown that Tic22 needs ATP, and not GTP, for its import into chloroplasts (Figure

17A). All these results point to the conclusion that import of Tic22 into the intermembrane space is relatively fast, but its processing is very slow and even after prolonged import duration the conversion from the precursor to the mature form never reaches more than around 20% *in vitro*.

In contrast to the stromal protein SSU and another investigated intermembrane space protein MGD1, Tic22 was not processed by the isolated stromal extract containing stromal processing peptidase (Figure 21A). This result, together with the low energy needs for its import, indicate that Tic22 might be imported through the outer envelope membrane, being immediately afterwards released to its final position in the intermembrane space. There, in the presence of higher ATP concentrations it could be stabilized and aquire the native conformation by interaction with intermembrane space chaperones. The question concerning its processing remains still unanswered. Mitochondria, like chloroplasts, are surrounded by two envelope membranes. Techniques have been developed to physically remove the mitochondrial outer envelope membrane. Mitoplasts, mitochondria of which the outer membrane has been selectively ruptured and/or dissolved, can be generated either by subjecting intact mitochondria to osmotic shock treatment (Daum et al., 1982) or by treating them with digitonin (Hartl et al., 1986). These two methods have been used successfully to study the localization and topology of mitochondrial inner envelope membrane proteins and the mechanism of mitochondrial protein import. Similar techniques to selectively remove the outer membrane of chloroplast envelopes are not yet available and the intermembrane space of chloroplasts has not been successfully isolated up to date. After the fractionation of chloroplasts it could be assumed that the stromal fraction contains a portion of the intermembrane space content. The stromal processing peptidase processed pSSU and pMGD1 with high efficiency. Since the stromal extract failed to process Tic22 it could be concluded that some other processing peptidase is required for processing of this protein. It is probable that Tic22 is processed in the intermembrane space of chloroplasts by a yet unknown peptidase or maybe by the same protease as iToc75 (Inoue et al., 2005). As mentioned in the introduction, Toc75 is processed by the SPP to the intermediate form (iToc75), which is cleaved to the mature Toc75 prior to insertion to the outer envelope, probably by the type I signal peptidase from the intermembrane space (Inoue et al., 2005).

Even when supposed that the intermembrane space content was isolated together with the stromal extract, it seems that an intermembrane space peptidase that would be responsible for processing of Tic22 was present there in too low amounts for successful processing to occur, or it could be associated to one of the envelope membranes and in this case was

isolated with the membrane fraction. To check the second possibility the radioactively labelled Tic22 was incubated with mixed envelope vesicles from *P. sativum* in the presence of 0.5% DeMa in 20 mM Hepes/KOH pH 8, 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Under the applied conditions no processing of Tic22 could be observed by the mixed envelopes (data not shown), leaving the question where the processing of Tic22 takes place unanswered.

To investigate whether Tic22 needs the presence of thermolysin-sensitive components on the chloroplast surface for its import, chloroplasts were pretreated prior to import with the protease thermolysin that removes receptors exposed to the cytosol. Import of Tic22 into the chloroplasts treated this way was diminished to 40 % (Figure 22), indicating that removed components are essential for binding and translocation of Tic22 into chloroplasts. Chloroplasts should be treated by such concentration of thermolysin that removes most of the surface exposed receptors, but in the same time does not disrupt significant amounts of chloroplasts. 1 mg thermolysin per mg chlorophyll was applied in the experiments and led to around 30-40% chloroplast loss during the reisolation step. It could be considered that not all receptors were removed under these conditions, what could explain the residual 40% of successful import of Tic22. A similar result was shown by Kouranov et al. (1999). Their investigation had shown that Tic22 is dependent on thermolysin sensitive components on the chloroplast surface. On the basis of competition experiments the same group suggested a new pathway for targeting of Tic22 to the intermembrane space, distinct from known chloroplast targeting pathways. The competition experiments performed in this work argue against this proposal. Increasing concentrations of the competitor protein pOE33, known to use the Toc- and Tic-translocon during its import into chloroplasts (Row and Gray, 2001), resulted in decreased import of Tic22. Even small quantities of pOE33 visibly inhibited Tic22 import, and the maximum of 10 µM pOE33 used in import reaction resulted in more than 70% inhibition of Tic22 import (Figure 23). The observed results clearly indicate that pOE33 and Tic22 use the same pathway for their import into chloroplasts, namely the Toc-complex on the general import pathway.

These results were confirmed by the competition experiments using Toc34ΔTM and by crosslinking experiments. Toc34ΔTM added in import mix competed with the endogenous Toc34 for binding of Tic22 (Figure 24), indicating that Toc34 recognizes and interacts with Tic22 at the initial step of its import into chloroplasts. Interaction between the soluble domain of Toc34 and precursor protein Tic22, but not its mature form (Figure 25), has shown again the specificity of this interaction and confirmed previously shown results. Chemical crosslinking and immunoprecipitation experiments clearly demonstrate that Tic22 binds to the

chloroplast surface by interaction with Toc34, Toc159 (data not shown) and Toc75, the members of the Toc-translocon. In immunoprecipitation experiment, antibody against atToc75(III) isoform precipitated Tic22, in contrast to α-Toc75(V). Crosslinking and immunoprecipitation, together with import competition experiments, indicate that Tic22 translocates through the Toc-complex to the intermembrane space. According to the low ATP-demands for its import, it seems that Tic22 is released from the Toc-complex to the intermembrane space of chloroplasts, where it reaches its final localization. Future experiments characterizing the exact site of processing, as well as investigation of the interaction of Tic22 with the components of the intermembrane space translocon, could clarify the events between the release from the Toc75 translocation channel and aquiring its final conformation. Crosslinking of Tic22 to Tic110 has been observed to a certain extent, what could be explained by their interaction on the intermembrane side of the inner envelope, as components of the Tic-translocon. The observed interaction does not mean that Tic22 uses Tic110 for further translocation because the stromal protein SSU, which is known to transverse the Tic-translocation channel, was found to be immunoprecipitated at much higher extent with Tic110 during its import.

7.3 Import and localization of MGD1 in the intermembrane space of chloroplasts

MGDG synthases from different plants have been extensively studied (Maréchal et al., 1993, Shimojima et al., 1997, Miège et al., 1999, Maréchal et al., 2000), but the properties of their import pathways into chloroplasts have not been shown yet. Most of MGD1 import experiments in this work have been performed paralelly to Tic22, expecting some similarities in their import pathway on the basis of their intermembrane space localization. Hirsch and Soll (1995) have observed that 80 mM K_iPO_4 had increased import of the inner envelope membrane protein of 96 kDa, but diminished import rates of pSSU. Import of Tic22 in this research was not affected by the addition of K_iPO_4 to any extent, in contrast to MGD1. Addition of 80 mM K_iPO_4 to the import reaction enhanced the import rate of MGD1 (Figure 16B). The observation that the mature MGD1 appears in two forms and that both forms behaved the same in response to various import conditions led to the conclusion that they represent two forms of the same mature protein – a lipid associated (smeared) one and a part pressed by the large subunit of RubisCO. The fact that the lipid-associated form was hardly

recognized by α-MGD1-P antibody (Figure 29) agrees with the results observed by detection of MGD1 in whole chloroplasts, where MGD1 was hardly or not at all detectable (results not shown). The reason of hard detectability could be the low abundance of MGD1 in the plastid envelopes (~1/1000 of total protein), as well as the lipid environment that could interfere with proper interaction between the antibody and the protein. When chloroplastic envelopes were tested for the presence of MGDG synthase by western blotting, envelope amount corresponding to at least 20-30 µg total protein had to be used for MGDG synthase signal to be detectable.

The existence of the cleavable presequence was confirmed already by Awai et al. (2001), who immunodetected the mature polypeptide of MGD1 in the envelope of chloroplasts from *Arabidopsis* leaves after cleavage of its transit peptide. In this work, comparison of the sizes of MGD1 detected by western blotting *in vivo* with the mature forms visible after its import into chloroplasts confirmed that MGD1 contains an around 11 kDa long presequence (as predicted by ChloroP) that targets the protein to chloroplasts where it is subsequently cleaved.

MGD1, like Tic22, is dependent exclusively on ATP for its import (Figure 17B). Although the processing of MGD1 has been observed even by performing import on ice, thermolysin protected forms started to appear after addition of 50 µM ATP and complete import was accomplished using 1 mM ATP (Figure 17C). MGD1 was processed by stromal extract (Figure 21). It was also imported more slowly than Tic22 in response to added ATP (Figures 18, 19C). The fast processing by stromal extract, the strong dependence on ATP for the complete translocation and the different ATP-dependence curve from Tic22 indicate that MGD1 most probably utilizes the import complexes from both envelope membranes. It translocates through the Toc-complex and, in contrast to Tic22, enters the Tic-translocon to enable the processing from the side of the stromal compartment to take place. To which extent MGD1 is exposed to the stroma during the processing could not be determined by the performed experiments. MGD1 is most probably never completely released from the inner envelope translocation channel, but rather exposes just its N-terminal transit sequence to the SPP, and is subsequently pulled back towards the intermembrane space (as seems to be true for iToc75, destined for the outer envelope).

Thermolysin pretreatment of chloroplasts had an influence on MGD1 import, but to a lesser extent than on Tic22. Thermolysin pretreatment decreased the import of MGD1 to around 50 %, indicating that MGD1 is dependent on thermolysin sensitive receptors on the chloroplast surface for its import. Competition for import with pOE33 indicates that MGD1

probably uses the Toc-translocon for its import into chloroplasts. As for Tic22, these results were confirmed by three further experiments: competition experiments using Toc34ΔTM, interaction of the preprotein with Toc34ΔTM and crosslinking and immunoprecipitation with the major components of the Toc-translocon. Toc34ΔTM competed with the endogenous Toc34 for binding of MGD1 (Figure 24), indicating that Toc34 recognizes and interacts with pMGD1 at the initial step of its import into chloroplasts. Only the precursor protein interacted *in vitro* with the soluble receptor Toc34ΔTM (Figure 25). MGD1 was found crosslinked to Toc34, Toc75 and to Tic110. For the crosslinking experiment, conditions that allowed only binding to the chloroplast surface were used. Therefore the interaction between MGD1 and Tic110 had a weaker character. Crosslinking of late import intermediates (for example after 2-5 minutes of import at 25ºC in the presence of 3mM ATP) could characterize import pathway of MGD1 further. Indeed, already by prolongation of the binding period, crosslinking to Tic110 could be observed (other components of the Tic-translocon have not been tested in this work). This result points to the previous proposal that MGD1 could use both Toc- and Tic-complexes for its import into chloroplasts.

Different localizations of MGDG synthases have been proposed in different plants. Enzymatic activity of MGDG synthase from spinach was found to be located on the inner membrane of the spinach chloroplast envelope (Block et al., 1983). Cline and Keegstra (1983) fractionated pea chloroplasts by flotation-centrifugation into thylakoids, soluble fraction and envelopes, that were further separated on a linear-density sucrose gradient. The gradient was assayed for galactosyltransferase, whose activity was detected in the outer envelope fraction and in one of the subfractions of the inner envelope, probably due to the contamination with the outer envelope. They proposed that MGDG synthase from pea associates to the outer envelope, but its presence from the inner envelope could not be excluded. In this work the localization of endogenous MGDG synthase in *P. sativum* have been investigated.

Treatment of the inner and outer envelope vesicles with 6 M urea gave distinct extraction results for MGDG synthase. The portion of the protein associated to the inner envelope was not extracted by 6 M urea (Figure 31, lanes 1 and 2), and the portion associated to the outer envelope was found mostly in the soluble fraction (Figure 31, lanes 7 and 8). Miège et al. (1999) analyzed the association of spinach MGD A with the inner envelope membrane by ionic and alkaline extractions. Although treatment of envelope membranes by 1 M NaCl had no effect on the enzyme association with the membrane, part of the protein was extracted by 0.1 M Na_2CO_3 pH 11 and all protein was extracted by 0.1 M NaOH. These

results suggested that soMGD A is neither a transmembrane nor a peripheral protein, but more likely is imbedded within one of the two leaflets of the inner envelope membrane. In this work 1 M NaCl had no influence on the association of pea MGDG synthase with the envelopes and 0.1 M Na_2CO_3 pH 11.5 extracted only a portion of the protein associated to the outer envelope. The observed results are similar to those obtained in spinach. It could be concluded that although preferentially associated to the outer envelope membrane, the portion of MGDG synthase from *P. sativum* interacts much stronger with the inner envelope, from where it could not be extracted either with 6 M urea, or with 0.1 M Na_2CO_3.

Thus, although the localization of MGD1 in chloroplasts is not entirely clear, it could be shown in this work that pMGD1 uses the general import pathway and reaches, at least partly, the stromal compartment before ending up in the intermembrane space. Taken together, it has been clearly demonstrated that proteins of the inner envelope and intermembrane space partly use the general import pathway, but their route to their final destination diverges at the inner membrane. Therefore, for chloroplasts the same picture begins to emerge that has been shown for mitochondria – that different import pathways exist for distinct protein classes.

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Curriculum vitae

Date and place of birth

11th March 1979, Zagreb, Croatia

Elementary school education

1985-1993, Zagreb

Secondary school education

1993-1997, Gymnasium for Mathematics and Natural Sciences (V. Gymnasium), Zagreb

Academic education

1997-2002 Study of Molecular Biology at the Department of Biology, Faculty of Science, University of Zagreb

1999-2000 Research on the thesis "Chloroplast isomerase TLP40 gene homologue *sll0408* has the important role in cyanobacterium *Synechocystis* PCC6803 biogenesis" awarded by The Rector award in 2000 (Performed at the Ruđer Bošković Institute, Zagreb, under the supervision of Prof. Dr. Nikola Ljubešić and Dr. Hrvoje Fulgosi)

2000-2002 Diploma degree research "Activity of retrotranspozon Tpv2 in *Arabidopsis thaliana L.*" (Performed at the Department of Molecular Biology, Faculty of Science, University of Zagreb, under the supervision of Prof. Dr. Dražena Papeš and Dr. Srećko Jelenić). Graduated in October, 2002

2002- Since November 2002, PhD thesis in the lab of Prof. Dr. Jürgen Soll at the Botanical Institute, Faculty of Biology, Ludwig-Maximilian University, Munich

Ehrenwörtliche Versicherung

Hiermit versichere ich dass die vorliegende Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Lea Vojta
München, den 14. 11. 2006

Erklärung

Ich habe zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen. Die Dissertation wurde keiner weiteren Prüfungskommision weder in Teilen noch als Ganzes vorgelegt.

Lea Vojta
München, den 14. 11. 2006