Function and Topology of Toc64, a subunit of the protein translocation machinery of the chloroplast outer envelope

Dissertation der Fakultät für Biologie
der Ludwig-Maximilians-Universität München

Vorgelegt von
Soumya Qbadou

München
30.06.2006

Tag der mündlichen Prüfung: 18.09.06

Gutachter:

1 Erstgutachter Pr. Dr. Jürgen Soll
2 Zweitgutachter Pr. Dr. Enrico Schleiff

Liebe ist das Einzige das wächst, wenn wir es verschwenden,
und das Morgen kann nur blühen,
wenn es im Gestern wurzelt und im Heute wächst.

Contents:

Abbreviations:

aa	amino acid
at	Arabidopsis thaliana
BN-PAGE	blue native-polyacrylamide gel electrophoresis
cBAG	Bcl2-associated anthanogene
EDTA	ethylenediaminetetraacetic acid
Fd	ferredoxin
GA	Geldanamycin
Hsp70	heat shock protein of 70kDa
Hsp90	heat shock protein of 90kDa
IE(V) or OE(V)	inner or outer envelope (vesicles)
is	intermembrane space localized
MDH	malate dehydrogenbase
NTT1	nucleotide transport protein 1
PEG-MAL	polyethylenglycol-maleimide
PC	plastocyanin
pOE33	preprotein of 33 kDa subunit of the oxygen evolving complex
pp	Pyscomitrella patens
pSSU	preprotein of SSU
ps	Pisum sativum
rlt	reticulocyte lysate translated
SDS-PAGE	SDS-polyacrylamyd gel electrophoresis
SSU	small subunit of RubisCO
Toc/Tic	translocon at the outer/inner chloroplastic envelope membrane
Tom/Tim	translocon at the outer/inner mitochondrial envelope membrane;
TPR	tetratricopeptide repeat
vs	Versus
wgt	Wheat germ translated

1. Summary

Precursor protein targeting toward surfaces of organelles is assisted by different cytosolic chaperones. The Toc translocon recognizes precursor proteins and facilitates their translocation across the outer envelope of chloroplasts. Toc64 is a subunit of the chloroplast protein import machinery. This work focuses on topological and functional properties of Toc64. The topological prediction of the protein by different programs revealed that Toc64 contains three transmembrane domains, which has been confirmed by the obtained biochemical an experimental results. It was demonstrated that the TPR domain of Toc64 is cytosolic exposed, whereas a second domain of about 30 kDa is exposed to the intermembrane space and protected by the chloroplast outer envelope, which is a part of the amidase and charged regions. Functional analysis demonstrated that Toc64 is a bi-functional preprotein receptor. First, the cytosolic exposed TPR is the docking site for Hsp90 bound precursor proteins. The Hsp90 is recognised by the clamp type TPR of Toc64. Hence, a novel mechanism in which chaperones are recruited for a specific targeting event by a membrane-inserted receptor is outlined. Second, the intermembrane space exposed domain allows the association of Toc64 with the Toc complex and is involved in precursor protein recognition and translocation across the intermembrane space. This domain also participates in the formation of the intermembrane space complex, which involves Toc12, isHsp70 and Tic22.

2. Zusammenfassung

Im Zytosol wird die Zielsteuerung von Vorstufenproteinen zu der äußeren plastidären Hüllmembran durch verschiedene zytosolische Chaperone unterstützt. Die Erkennung und die Translokation der Vorstufenproteine über die äußere Hüllmembran wird durch den Toc-Komplex katalysiert. Der Schwerpunkt dieser Arbeit war die Untersuchung struktureller und funktioneller Eigenschaften des Toc64 Proteins, einer Untereinheit des Toc-Komplexes. *In silico* Topologie-Vorhersagen für Toc64 ergaben drei Transmembranbereiche, welche auch experimentell bestätigt werden konnten. Die topologische Analyse ergab, daß die TPR-Domäne von Toc64 auf der zytosolischen Seite exponiert ist, während ein protease-resistentes Fragment mit einem apparenten Molekulargewicht von 30 kDa in den Intermembranraum hineinragt. Dieses Fragment beinhaltet Teile der Amidase- sowie der geladenen Region. Durch funktionelle Untersuchungen wurde gezeigt, dass Toc64 ein bifunktioneller Vorstufenprotein-Rezeptor ist**.** Einerseits bildet die zytosolisch exponierte TPR Domäne die Erkennungsstelle für Hsp90 gebundene Vorstufenproteine. Hsp90 interagiert dabei mit der „Clamp-typ“ TPR Domain des Toc64 Proteins. Demzufolge wurde ein neuer Mechanismus, in dem Chaperone für eine spezifische Zielsteuerung und Erkennung der Vorstufenproteine durch membrane-inserierte Rezeptoren notwendig sind, gefunden. Andererseits ermöglicht das im Intermembranraum exponierte 30 kDa Fragment die Assoziation von Toc64 mit dem Toc-Komplex und ist involoviert in der Erkennung und Translokation der Vorstufenproteine. Dieses Fragment ist auch an der Bildung des Intermembranraum-Komplexes, welches Toc12, isHsp70 and Tic22 enthält, beteiligt.

3. Introduction

Plastids are a heterogeneous family of organelles found ubiquitously in high plants and algae. Most prominent amongst these are the chloroplasts, which are responsible for the light-harvesting and carbon-fixation reactions of photosynthesis, as well as for the synthesis of many essential metabolites, such as fatty acids and amino acids. Like mitochondria, plastids entered the eukaryotic lineage through endosymbiosis. They are thought to be of monophyletic origin, and to have evolved from an ancient photosynthetic prokaryote similar to present-day cyanobacteria (Palmer, 2000; Leister, 2005). They are separated from the surroundings cell by two membranes, which represent an effective barrier for metabolites and proteins. Chloroplasts are complex organelles comprising six distinct suborganellar compartments: they have three different membranes (the two envelope membranes and the internal thylakoid membrane), and three discrete aqueous compartments (the intermembrane space of the envelope, the stroma and the thylakoid lumen). As all plastids within an organism contain the same limited complement of about 100 genes, it is the imported proteins that define the developmental fate of the organelle. Therefore, most chloroplast proteins are nuclear encoded, synthesized on cytosolic ribosomes as precursor proteins, and posttranslationaly imported into the organelle via translocation complexes in the outer and inner envelope membrane of chloroplast, in order to maintain various biochemical functions (Soll and Schleiff, 2004; Kessler and Schnell, 2004, Jarvis and Robinson, 2004). The import comprises a complex system of cytosolic targeting, translocation through the two envelope membranes and a subsequent intra-organellar sorting. Proteins destined for plastid import can be divided into at least two classes (Soll and Schleiff, 2004). The first class includes proteins, which are translated on cytosolic ribosomes as so called precursor proteins with a cleavable N-terminal transit peptide. The transit peptide provides essential and sufficient information for subsequent targeting towards and translocation into the plastid. This class encloses the majority of plastid proteins (Soll and Schleiff, 2004). The proteins that belong to the second class do not contain any cleavable transit peptide. Members of this class are most of the outer envelope proteins and at least two inner envelope membrane proteins (Schleiff and Klösgen, 2001; Miras et al., 2002; Nada and Soll, 2004).

The first step in chloroplast protein translocation is the transport through the cytosol. While the translocation process itself is understood in some molecular detail, the mechanism by which the preproteins are transferred from the cytosol to the Toc translocon (translocon of the outer envelope of chloroplasts) remains elusive. In case of chloroplasts it is proposed that

phosphorylation of some transit peptides (like the small subunit of RubisCO (pSSU)) enhances the import rate presumably through interaction with 14-3-3 proteins, which form a guidance complex with Hsp70 proteins and potential other factors (May and Soll, 2000). Thus, phosphorylation of preproteins is not essential for import into chloroplasts, but was suggested to accelerate the targeting and not the import process of a specific set of preproteins (Nakrieko et al., 2004) (Figure 1).

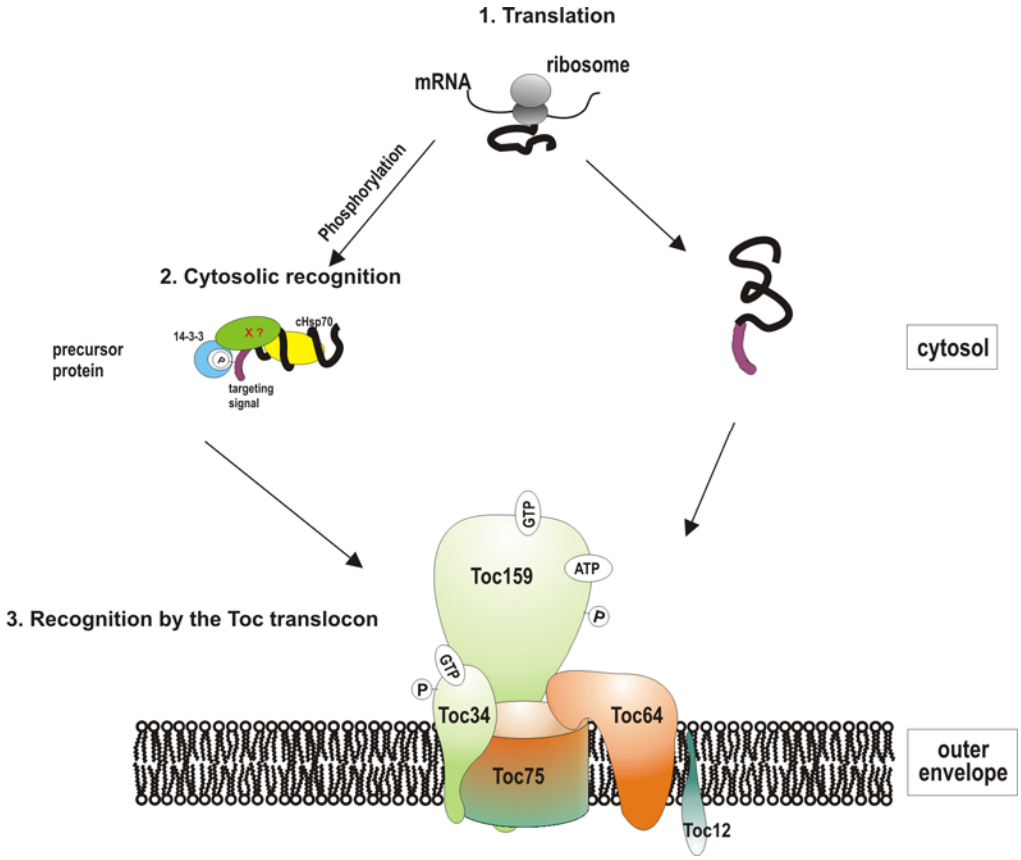

Figure 1: The cytoslic targeting of chloroplast precursor. In the cytosol, preproteins with an amino-terminal presequence or transit sequence can form a cytosolic guidance complex upon phosphorylation in the cytosol. This guidance complex consists of an HSP70 (heat shock protein-70) chaperone, 14-3-3 proteins and other unknown factors. All these complexes bind to TOC receptors in a GTP-dependent manner. Other preproteins are targeted to the outer envelope without or by assisting of unknown cytosolic factors.

The second step is the recognition of precursor proteins at the outer envelope membrane by the Toc translocon. Until now five subunits of the Toc complex have been identified, namely Toc159, Toc75, Toc34, Toc64, and Toc12 (Soll and Schleiff, 2004). Toc75, Toc159 and Toc34 represent the core proteins in the Toc complex. They form a stable core complex of ~550 kDa, which has a stoichiometric ratio of 4:4:1 for Toc34:Toc75:Toc159 (Schleiff et al.,

2003a). The translocation of the preproteins across the inner envelope membrane requires proteins of the Tic translocon (translocon of the inner envelope of chloroplasts). Several Tic subunits have been identified, and these are: Tic110, Tic62, Tic55, Tic40, Tic32, Tic22 and Tic20. Tic110 is the major protein in the Tic complex. Tic22 is peripherally associated with the outer face of the inner membrane. It might have a vital role in the translocation of the preproteins across the intermembrane space (Kouranov et al., 1998). Therefore, Tic22 is the only intermembrane space protein involved in preprotein translocation reported so far (Becker et al., 2004a) (Figure 1).

The Toc/Tic complex facilitates the passage of the translated preproteins directed to the stroma across the envelopes. Once it arrives in the stroma, the transit peptide is cleaved off by a stromal metallo-peptidase called stromal processing peptidase (SPP), and subsequently degraded by a second metallo-peptidase (TTP) (Oblong and Lamppa, 1992; VanderVere et al., 1995; Richter and Lamppa, 1998).

3.1 Involvement of chaperones in preprotein translocation

Chaperones are phylogenetically one of the most conserved families of proteins found in all organisms, from prokaryotes, yeasts and plants to animals. In their classical function chaperones assist in protein folding and protein translocation. They prevent misfolding and aggregation of proteins and facilitate refolding of denaturated proteins (Georgopoulos et al. 1993; Bukau et al. 1998).

Cytosolic targeting of precursor proteins to the chloroplast surface involves cytosolic Hsp70s and 14-3-3 protein so called “guidance complex” and maybe other so far unknown components (May and Soll, 2000). A subset of precursor proteins of chloroplast contains a phosphorylation motif within the N-terminal transit peptide, which shares similarities to 14-3-3 binding sites. These proteins are bound by the “guidance complex”, which stimulate transfer to the chloroplast surface. It has been reported that the association of chaperones of the Hsp70 type is mediated by the nature of the transit peptide (Rial et al., 2000). Interestingly, the 14-3-3 protein recognises the presequence of the precursor protein as well. The docking site of the guidance complex, the stage and the mode of dissociation remains unknown.

The targeting of preproteins to the surface of mitochondria is comparable to chloroplasts. The newly synthesized mitochondrial preproteins contain specific targeting signals and are usually bound by factors which maintain the preproteins in a translocation-competent conformation. These are chaperones of the Hsp70 family as well as specific factors like MSF (Mitochondrial import Stimulation Factor) (Hachiya et al., 1993), which is a 14-3-3 protein different from

that of the "guidance complex" for chloroplasts (May and Soll, 2000). This "MSF" presumably recognizes mitochondrial targeting signals (Murakami et al., 1988; Komiya et al., 1996; Mihara et al., 1996). Recently, it was shown that the chaperone Hsp90, which has been thought to act largely on signal transducing proteins, in cooperation with Hsp70, mediates the targeting of a subset of mitochondrial preproteins to the translocon Tom70 in mammals (Young et al., 2003). These chaperones interact with precursor proteins depending on the presence of specific targeting information within the primary structure of the protein (Young et al., 2003). Generally, it remains unknown whether Hsp70 binding is limited to the transit sequence or comprises further targets in the mature domain, since cytosolic Hsp70 is generally involved in the folding of newly synthesized proteins (Bukau et al, 2000; Young et al, 2004), and its association seems to be required to keep the preproteins in an import competent unfolded state and prevent the enzymatic activity of proteins in a wrong cellular compartment (Deshaies et al., 1998, Weagemann et al., 1990).

3.2 Recognition and transfer of preproteins at the chloroplast surface

The translocon for preprotein transport across the outer envelope consists of three precursor binding proteins with known or proposed functions, Toc34, Toc159 and Toc64 (Soll and Schleiff, 2004; Kessler and Schnell, 2004). Toc159 and Toc34 are GTPases regulated by phosphorylation. Toc34 acts as initial receptor within the Toc core complex composed of Toc34, Toc159 (Schleiff et al, 2003b; Becker et al, 2004b) and the channel-forming Toc75 (Hinnah et al., 2002; Svesnikova et al., 2000). Toc34 is active for preprotein and GTP binding in a nonphosphorylated state, and can be inactivated by phosphorylation (Jelic et al., 2002). $Toc34_{GTP}$ binds the phosphorylated C-terminus of preproteins with high affinity (Jelic et al 2002; Schleiff et al., 2002). Preprotein association enhances GTP hydrolysis and subsequently preproteins are released to the next translocon subunit Toc159. The receptor can then be recharged with GTP and enter a new recognition cycle. In an action that also requires GTP hydrolysis, Toc159 initiates the transfer of the preprotein through the Toc75 channel (Becker et al., 2004b). Toc159 is crucial for the import process (Bauer et al., 2000, Schleiff et al., 2003b, Smith et al., 2004).The essential role of Toc159 is underlined by a lethal phenotype of a T-DNA insertion into *at*Toc159 gene (Bauer et. al., 2000). This further indicates that the four paralogues of this receptor found in *A.thaliana* namely, Toc159, Toc132, Toc120, Toc90 have a specialised function in organelle biogenesis (Xiong and Bauer, 2002). In line with this finding, it was proposed that Toc159 paralogues exhibit different substrates specifity (Ivanova et al., 2004, Kubis et al., 2004).

Arabidopsis thaliana encodes two isoforms of Toc34 with similar function, namely atToc33 and atToc34 (Soll and Schleiff, 2004). AtToc33 seems to be the functional analogue of psToc34 (Jelic et al., 2003). The ppi1 mutant, a knockout of atToc33, has a pale phenotype and retarded chloroplast development. However, later in development, plants partially recover and are able to grow photoautotrophically on soil. This phenotype is probably due to the presence of a homologue of atToc33 in *Arabidopsis thaliana,* which is atToc34 (Gutensohn et al., 2000; Jarvis et al., 1998). Both functional subunits revealed different affinities for distinct types of preproteins. AtToc33 mainly recognises photosynthetic preproteins (Jelic et al., 2003), whereas atToc34 is involved in the import of nonphotosynthetic chloroplast proteins (Kubis et al., 2003). In contrast, Toc159 is essential for the import process (Bauer et al, 2000) because it facilitates the translocation event of preproteins (Schleiff et al, 2003a).

3.3 The Toc64 protein

In contrast to the GTPases, not much is known about Toc64. Toc64 is dynamically associated with the Toc core complex in pea (Sohrt and Soll, 2000, Becker et al., 2004a). It is not purified with the core complex, but the protein can be cross-linked to several subunits of the Toc complex (Sohrt and Soll, 2000). The Arabidopsis genome contains at least three proteins with similarities to Toc64, thereby suggesting that they are functional homologues (Jackson-constan and Keegstra, 2001). All three genes appear to be expressed *in vivo*, but only atToc64-III was identified on a protein level in chloroplast membranes so far (Ferro et al., 2002; Chew et al., 2004). AtToc64-V, on the other hand, was found to be targeted to mitochondria (Chew et al., 2004).

Recent work suggested that Toc64 contains an N-terminal transmembrane region, which is essential and sufficient for targeting to chloroplasts (Lee et al., 2004). Furthermore, it was documented that the N-terminal transmembrane region has a N_{in}-C_{out} orientation (Lee et al., 2004). In addition, the N-terminal hydrophobic domain of the two isoforms PpToc64-1 and -2 of *Physcomitrella patens* was necessary for interaction with chloroplasts, consistent with this region containing the transmembrane domain (Hofmann and Theg, 2005). Both proteins are not extractable after their insertion into chloroplasts and both remained accessible to the external protease, which leads to the suggestion of an entirely cytosolic exposed protein conformation (Hofmann and Theg, 2005). Toc64 can interact with Toc12, a component of the extended Toc translocon situated at the intermembrane space (Becker et al., 2004a). Toc12 contains a J-domain, which reaches into the intermembrane space. This domain is common to a family of DnaJ proteins and is required for the interaction of these proteins with Hsp70.

Consequentially; Toc12 was shown to interact with an Hsp70 homologue in the intermembrane space (Marshall et al., 1990). Together with Toc64 and Tic22, they might form a complex designed to assist in the transfer of precursor proteins across the intermembrane space. The evidence for the specific function of the Toc64 biochemical analysis will be presented in this work.

Primary sequence analysis of Toc64 revealed three domains (Sohrt and Soll, 2000). The first motif exhibits homology to prokaryotic and eukaryotic amidases, the enzymatic function as an amidase seems to be inactivated due to a point mutation at the position 170 (Ser → Gly) in its active site. The second motif includes a charged region, and is followed by a C-terminal third motif the threefold repeated TPR-motif (Sohrt and Soll, 2000). The TPR motifs share some similarity to other TPR domains in proteins acting as cofactors of Hsp90 and Hsp70 chaperones or of the mitochondrial protein import receptor Tom70. The TPR motif is a 34-amino acid consensus sequence that mediates protein-protein interactions in diverse cellular pathways (Ratajczak et al., 1996). Peptide regions composed of three TPR motifs are organized into a super-helical structure (Scheufler et al., 2000). The Toc64 TPR motif shares some similarity to the motifs found in Hop (Hsp70/90 organizing protein) which acts as cofactors of Hsp90 and Hsp70 chaperones or in the mitochondrial protein import receptor Tom70 (Young el al., 1998; Ramsey et al., 2000, Young et al., 2003). Hop recognizes the C-terminal sequences of Hsp90 and/or Hsp70 through specialized TPR domains (Scheufler et al., 2000). Both chaperones share a conserved C-terminal EEVD sequence that binds the central groove of the TPR domain (Demand et al., 1998; Young et al., 1998). The specificity for either Hsp70 or Hsp90 is determined by hydrophobic contacts with neighbouring residues (Scheufler et al, 2000). It was demonstrated that the TPR domain of Tom70 mediates the association of chaperone affiliated preproteins (Young et al. 2003). The similarity of the TPR domains of Tom70 and Toc64 is in line with the observation that one member of the Toc64 protein family in *A. thaliana* seems to replace Tom70 in plant mitochondria (Chew et al, 2004). Since, no homologue with any significant sequence identity to Tom70 can be detected in the Arabidopsis genome (Lister et al., 2003). However, the function of the TPR domain in Toc64 is still elusive.

3.4 The aim of this work

Toc64 is a component of the Toc translocon. To assign functional properties to single domains of Toc64, the topology of the protein in chloroplast membranes was investigated assisted by *in silico* analysis, to produce a topology model of Toc64. The evidence for the

existence of an intermembrane space exposed region of Toc64 has to be demonstrated, and its function in intermembrane space complex formation and preprotein recognition had to be explored. The function of chaperone docking and preprotein recognition by the TPR domain of Toc64 was a further question to be investigated. The results are finaly implemented into the current model of preprotein recognition and translocation.

4. Materials

4.1 Chemicals

If not otherwise noted, all chemicals were purchased from Sigma Aldrich (München, Germany), Roth (Karlsruhe, Germany), Roche (Penzberg, Germany) or Merck (Darmstadt, Germany). N-decylmaltoside was supplied from Glycon GmbH (Luckenwalde, Germany). Radio-labelled amino acids and nucleotides were obtained from GE-Healthcare (Freiburg, Germany).

4.2 Enzymes, kits and peptides

Enzymes for cloning, such as restriction endonucleases, T4-DNA-ligases and Taq-polymerases, were obtained from Roche (Penzberg, Germany), MBI Fermentas (St. Leon-Rot, Germany) or Qiagen (Hilden, Germany). Triplemaster-mix polymerase was purchased from Eppendorf (TripleMaster PCR System, Hamburg, Germany) and *Pfu* DNA polymerase from Promega (Mannheim, Germany). Thermolysin, proteinase K and chymotrypsin were purchased from Roche (Penzberg, Germany), trypsin from Sigma Aldrich (München, Germany) and RNase from GE-Healthcare (Freiburg, Germany). For DNA purification with high yield the Plasmid Midi Kit from Macherey and Nagel (Düren, Germany) was used, whereas for DNA purification on a smaller scale the Silica Spin Kit from Biometra (Göttingen, Germany) was utilised. Purfication of DNA fragments from agarose gels and purification of PCR products were carried out using QIAquick purification Kits provided by Qiagen (Hilden, Germany). *In vitro* translation was performed with reticulocyte or wheat germ extract Translation Kit supplied by Promega (Mannheim, Germany). Protease inhibitor cocktail tablets were purchased from Promega (Mannheim, Germany). The peptides CP of pSSU, PP1, PP2 of the phosphate carrier and P90 of human Hsp90 protein were synthesised at the Department of Peptide and Protein Chemistry at the Charite (Berlin, Germany).

4.3 Plant material and growth conditions

Seedlings of *Arabidopsis thaliana* were grown either on soil or on MS-plates (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose as described before (Kroll et al., 2001). In both cases the plants were grown in a climate chamber at 20°C with a 14h / 10h daylight cycle. Prior to illumination, plates were placed for 2 days at 4 °C to induce germination.

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

4.4 DNA primers

All DNA primers used in PCR reaction were ordered from MWG-Biotech (Ebersberg, Germany) or Quiagen (Hilden, Germany).

4.5 Vectors and E.coli strains

pET21d	Stratagen (La Jolla, USA)	(Studier und Moffatt, 1986)
pSP65	Promega (Madison, USA)	(Studier und Moffatt, 1986)
pGEX-4T3	Amersham (Freiburg, Germany)	
pBluscript	Stratagen (La Jolla, USA)	(Short et al., 1988)
pK7FWG2	VIB/ Ghent University (Ghent, Belgium)	
DH5α	GibcoBRL (Eggenstein, Deutschland)	(Woodcock et al., 1989)
Bl21(DE3)	Novagen (Madison, USA)	(Studier und Moffatt, 1986)

4.6 Membranes

Nitrocellulose membranes (Protran BA-S83, 0.2µm) were purchased from Schleicher and Schüll (Dassel, Germany).

4.7 Antibodies

In general primary antibodies against Toc64, Toc34 and 14-3-3 were produced by injection of recombinantly expressed proteins into rabbits (Vojta et al., 2004). Antibodies against Hsp90 and Hsp70 were purchased from StressGen (Biomol, Hamburg, Germany) and antibody against GFP from *Aequorea victoria* was from Roche (Penzburg, Germany). Secondary antibodies against rabbit and rat IgG conjugates from goat were purchased from Sigma (München, Germany).

4.8 Columns and column materials

The Ni^{2+}-NTA column material was supplied by Qiagen (Hilden, Germany), the AF-Tresyl Toyopearl 650M for N-terminal protein coupling was obtained from Tosoh Bioscience (Stuttgart, Germany). The thiol-activated propyl sepharose and thiol-activated sepharose were obtained from Sigma (München, Germany). Protein-A sepharose was supplied by GE-Healthcare (Freiburg, Germany).

5. Methods

5.1 Molecular biological methods

5.1.1. Standard methods

Bacterial strain culturing and transformation with DNA were performed according to standard protocols (Sambrook et al., 1989). The preparation of the competent cells for DNA transformation was based on the methods described by (Hanahan et al., 1985). Isolation, restriction, ligation and agarose gel electrophoresis of DNA were performed according to the standard procedures (Sambrook et al., 1989).

5.1.2 Cloning

Construct	Vector	Organism	Amino acid	Origin
Toc64	pET21d	*P. sativum*	full length (593aa)	Sohrt and Soll, 2000
Toc64-ΔTPR	pET21d	*P. sativum*	1-476	Sohrt and Soll, 2000
Toc64-TPR	pET21d	*P. sativum*	477-593	Sohrt and Soll, 2000
$Toc64TPR_{N516A}$	pET21d	*P. sativum*	477-593	This work
$Toc64TPR_{R550A}$	pET21d	*P. sativum*	477-593	This work
atToc64III	pBluscript	*A. thaliana*	full length (589aa)	This work
atToc64III-ΔTM	pBluscript	*A. thaliana*	21-589	This work
atToc64III-ΔA	pBluscript	*A. thaliana*	1-21, 205-589	This work
atToc64III-ΔC	pBluscript	*A. thaliana*	1-204, 398-589	This work
atToc64III-ΔAC	pBluscript	*A .thaliana*	1-21, 398-589	This work
atToc64III-ΔTPR	pBluscript	*A .thaliana*	1-474	This work
atToc64III-Δ1	pBluscript	*A .thaliana*	1-437	This work
atToc64III-Δ2	pBluscript	*A .thaliana*	1-250	This work
pOE33	pET3c	*P. sativum*	full length	Waegemann and Soll, 1996
pOE33	pGEM4Z	*T. aestivum*	full length	Waegemann and Soll, 1996
pSSU	pET21d	*N.tabacum*	full length	Waegemann and Soll, 1996
pSSU	pSP65	*P.sativum*	full length	Waegemann and Soll, 1996
pNTT1	pET16b	*A. thaliana*	full length	Prof. Neuhaus (Kaiserslautern, Germany)
pMDH	pSP65	*P.sativum*	full length	Prof. Neuhaus (Kaiserslautern, Germany)
pPC1	pSP65	*P.sativum*	full length	Prof. Klösgen (Halle, Germany)
pFd	pSP65	*A. thaliana*	full length	Prof. Klösgen (Halle, Germany)
14-3-3	pET15b	*A. thaliana*	full length	AG Soll
Toc34ΔTM	pET21b	*P.sativum*	1-252	Jelic et al., 2002
pOE33-GFP	pK7FWG2	*T. aestivum*	full length	This work
pSSU-GFP	pOL-GFP	*N.tabacum*	full length	AG Soll

Table1. List of constructs used in this study**.** The name (column 1), the vector used for cloning (column 2), the biological source (column 3), the purpose of cloning (column 4) and the source are given for each construct.

Restriction sites for cloning of DNA fragments into plasmid vectors were generated by polymerase chain reaction (PCR) (Saiki et al., 1989). PCR reaction was carried out as recommended by polymerase suppliers for Triplemaster-mix polymerase or *Pfu* DNA

polymerase. The atToc64-III construct was isolated from *Arabidopsis* cDNA library and cloned in pBluscript. AtToc64-III deletion-mutants for *in vitro* translation were generated utilizing this constructs as template, truncations were generated by PCR with internal primers. PCR products were cloned into pBluescript and controlled by sequencing. Toc64 from *P. sativum* was used as template to generate constructs for expression, (Sohrt and Soll, 2000). Constructs were subsequently cloned into pGEX-4T or pET21d and controlled by sequencing (Table 1). Point mutations were introduced by QuickChange site-directed mutagenesis method (Bedwell et al., 1989). In brief: the QuikChange site-directed mutagenesis method is performed using *Pfu*-DNA polymerase. *Pfu*-DNA polymerase replicates both plasmid strands with high fidelityll and without displacing the mutant oligonucleotide primers. The oligonucleotide primers containing the desired mutation are complementary to opposite strands of the vector. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion. The pOE33-GFP construct was generated using Gateway cloning technology according to manufacturer's recommendation (Invitrogen, Karlsruhe, Germany).

5.1.3 Schematic representation of Toc64 and Toc34 Constructs

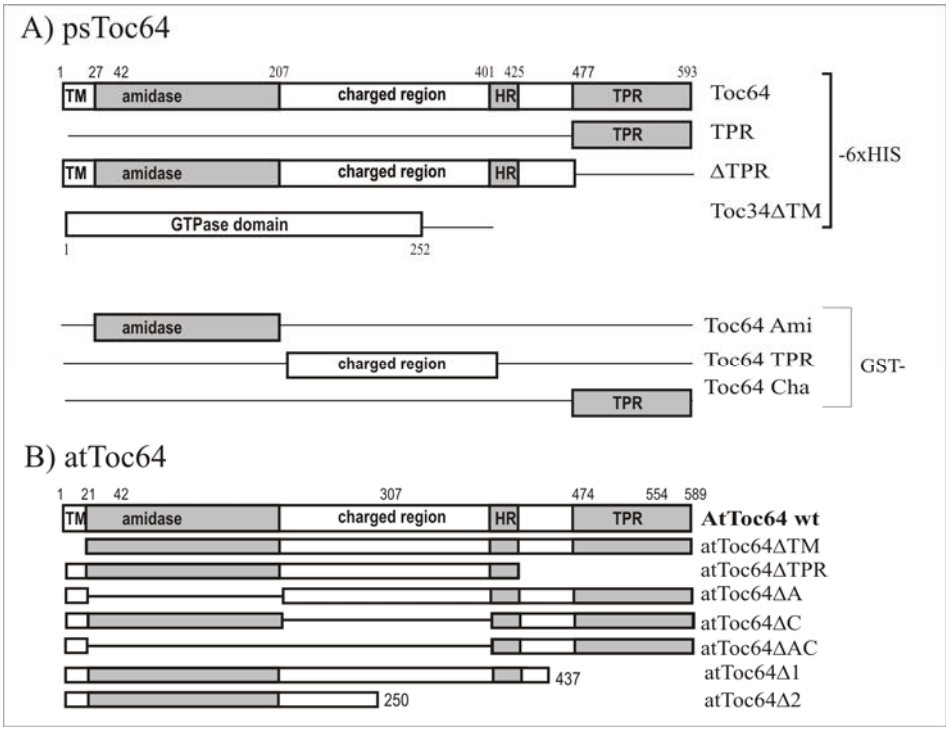

Figure 2. ps: *Pisum sativum*; at: *Arabidopsis thaliana*

5.1.4 RNA Isolastion from *Arabidopsis thaliana* and RT-PCR

mRNA was isolated from leaves using the RNeasy Plant Mini Kit according to the recommendation of the manufacturers (Qiagen, Hilden, Germany).

The RT-PCR (reverse transcription PCR) reaction was conducted using Two-Step MMLV RT-PCR Kit according to manufacturer's recommendation (Promega, Mannheim, Germany).

5.2 Biochemical methods

5.2.1 *In vitro* transcription and translation

The *in vitro* transcripition of inearized plasmids was carried out in a reaction volume of 50µl containing transcription buffer (10mM DTT, 100U Rnase inhibitor, 0.05% (w/v) BSA, 0.5mM ATP, CTP, and UTP, P´-5´-(7-Methyl)-Guanosin-P^3-5´-Guanosin (Cap) and 10U RNA polymerase. The reaction mixtrue was incubated for 15 min at 37°C. Finally, 12 mM GTP was added and transcription mixture was incubated for another 60 min. The radioactive labelled proteins were generated using *in vitro* translation of mRNA in reticulocyte lysate translation following the manufacturer´s instructions. 150µCi of ^{35}S-methionine/cysteine mixture were added for radioactive labelling.

The coupled *in vitro* transcription/translation was carried out using the TNT Kit (a coupled system from Promega (Mannheim, Germany)) according to the recommendation of the manufacturer.

5.2.2 Isolation of intact chloroplast and their fractionation

Isolation of intact chloroplasts from leaves of 10-12 day old garden pea and chloroplast fractination was performed as described in (Schleiff et al. 2003). *Arabidopsis thaliana* chloroplasts were isolated from 3-4 week old plants as described in (Arronson and Jarvis, 2002).

5.2.3 Import of preproteins into isolated chloroplast

For *in vitro* import assays into isolated chloroplasts, the postribosomal supernatant of radioactive-labelled proteins after centrifugation (10 min / 250,000 x g / 4°C) was used. After chloroplast purification the chlorophyll concentrations was determined as described by (Arnon, 1949). A standard import assay into chloroplasts equivalent to 20 µg chlorophyll was performed in 100 µl import buffer (10 mM methionine, 10mM cysteine, 20 mM potassium gluconate, 10 mM $NaHCO_3$, 3 mM $MgSO_4$, 330 mM sorbitol, 50 mM Hepes/KOH pH 7.6) containing up to 10% of *in vitro* translated ^{35}S labelled protein. Import was initiated by

addition of organelles to import mixture and transfer to 25°C. The reaction was stopped after15 min or at the indicated times. Intact chloroplast were reisolated through a Percoll cushion (40% Percoll in 330 mM sorbitol, 50 mM Hepes/KOH, pH 7.6) washed in 330 mM sorbitol, 50 mM Hepes/KOH, pH 7.6, 3 mM $MgCl_2$, and used for further treatments as described (Schleiff et al., 2001).

5.2.4 Protease treatment and extraction of outer envelope vesicles or chloroplasts

Purified envelope membranes of chloroplasts or intact chloroplasts were treated with proteases as described (Sveshnikova et al., 2000). After inhibition of the protease, chloroplasts were disrupted by incubation in 20 mM Hepes pH 7.5 for 5 min on ice followed by membrane recovery by centrifugation at 100 000 x g for 10 min at 4°C. Proteins of the outer envelope or of the chloroplast membrane fraction were extracted by Methanol / chloroform as described (Schleiff et al., 2001) and separated by SDS-PAGE.
Extraction of associated proteins was accomplished by the incubation of isolated chloroplasts or outer envelope vesicles with 100 mM Na_2CO_3 pH 11.4 for 20 min at 4°C. After re-isolation of the membrane vesicles by centrifugation (256 000 x g, 10 min, 4°C) the soluble and membrane fractions were subjected to SDS-PAGE analysis and immunoblotting.

5.2.5 Pegylation assay

After *in vitro* import assays chloroplasts were diluted with (2 mM EDTA, 20 mM PEG-MAL) to give a final PEG-MAL (PEG-MAL, MW 5000; Shearwater.Inc, Holualoa, US) concentration of 10 mM. After 20 min incubation at 4°C the crosslinking reaction was quenched by addition of 100 mM DTT. Chloroplasts were subsequently treated as indicated in the figure legends and the protein content analyzed by SDS-PAGE using MOPS running buffer according to the protocol of the manufacturer for NuPAGE-Novex Gels (BIO.COM, Canada).

5.2.6 Heterologous protein overexpression and purification

Expression of chimerical constructs was performed in *E.coli* strain BL21 (DE3). pOE33, pSSU, Toc34ΔTM and GST-fusion constructs of Toc64 (GST-Ami, GST-Cha, GST-TPR) were expressed and purified as described in (Weageman and Soll, 1996, Jelic et al., 2000, Nada and Soll, 2004, Sohrt and Soll, 2000). In brief, plasmids containing cDNA encoding for Toc64 constructs were transformed into BL21(DE3) cells and grown in 2YT/A media to an OD_{600} of 0.4 at 37°C. Subsequently, the culture was cooled to 12°C before the expression was

induced by addition of 1mM IPTG (final concentration). After 12 hours cells were harvested, resuspended in (100 mM Tris/HCl pH 8, 200 mM NaCl, 10 mM β-Mercaptoethanol) and lysed by 1200 psi pressure in a french press. Large molecules like DNA or membrane shreds were destroyed by 10 seconds of sonication. Subsequently, insoluble proteines were removed by centrifugation (20000 x g, 30 min, 4°C). The supernatant was incubated with Ni-NTA for 45 min at 20°C. Subsequently, the column was washed with 15 column volumes 50 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM β-Mercaptoethanol and 5 column volumes of 50 mM Tris/HCl pH 8, 1 M NaCl, 1 mM β-Mercaptoethanol. Bound proteins were eluted by 250 mM imidazol, 50 mM Tris/HCl pH 8, 150 mM NaCl, 1 mM β-Mercaptoethanol. The eluted proteins were pooled and directly dialysed against 5mM Hepes KOH pH 7.6, 50 mM K-Acetat, 5% Glycerol and 1mM β-Mercaptoethanol over night at 4°C. Precipitated proteins were removed by centrifugation (25000 x g, 5 min, 4°C).

5.2.7 Analysis of protein-receptor interaction

5.2.7.1 Affinity chromatography with receptor coated Ni-NTA

For analysis of the interaction of preproteins with Toc proteins, the protein-loaded Ni-NTA resin (final amount of 50 pmol of each Toc protein/100µl assay) was equilibrated with assay buffer (20 mM Hepes, 50 mM KCl, 5 mM $MgCl_2$). The competitors were added as indicated. Then the resuspended resin was transferred into 1 ml Mobicol columns. A mixture of ^{35}S-labelled preproteins in assay buffer (maximum 7% (v/v) wheat germ or rabbit reticulocyte lysate) was added, and the resin was resuspended. The binding was performed at 4°C for 30 min. After sufficient washing with binding buffer the bound proteins were eluted with buffer containing 500 mM NaCl. Flow through, wash and eluted fractions were subjected to SDS-PAGE analysis.

The binding of wheat germ, pea or yeast chaperones to Toc64 was performed at 4°C for 30 min. 5µmM purified Toc64 full length, Toc64TPR or Toc64TPR with point mutations proteins were coupled to the column material, and incubated with either wheat germ, pe*a* or yeast extract in binding buffer (20mM Hepes, 50mM KCl, 5mM $MgCl_2$). The bound proteins were eluted by 500 mM NaCl in binding buffer (Young et al., 2003). The eluted samples were subjected to SDS-PAGE analysis and immunoblotting.

5.2.7.2 Chromatography using protein coupled to Toyopearl matrix

Preproteins and Toc64 constructs were coupled to Toyopearl affinity matrix as described (Schleiff et al., 2003). The concentration of bound on Toyopearl column material was

1 mg/ml. Wgt S^{35}-labelled pOE33 or pSSU were incubated with a Toc64 matrix. The binding was performed as in (5.2.7.1). After sufficient washing, as controlled by subjecting the wash steps to SDS-PAGE analysis, bound proteins were eluted by increasing amounts of expressed Toc64TPR, Toc64ΔTPR, pSSU or pOE33 proteins. Eluted proteins were visualised by autoradiography.

After Thermolysin treatement and carbonate extraction outer envelop vesicles were incubated with pSSU or pOE33 coated matrices as in (5.2.7.1). After sufficient washing bound proteins were eluted with 8 M urea from Toyopearl column material.

5.2.7.3 Chromatography with thiol sepharose coupled substrates

For binding site analysis peptides indicated (4.2) containing a carboxyl terminal cystein were covalently coupled via a C-terminal cystein to thiol-activated Sepharose over night at 4°C to a final concentration of 0.6mg/ml column resin in (0.1M Tris/HCl pH 7.5, 0.5M NaCl, 1mM EDTA). After washing and controlling the coupling by measuring protein concentration at OD_{595}, the matrices were incubated with isolated receptor proteins in binding buffer (20mM Tricine/KOH pH 7.6, 0.1mM DTT, 5% glycerol, 0.05% TX-100, 50mM NaCl, 2mM $MgCl_2$, 0.1%BSA) for 30min at RT. After binding the column was washed sufficiently with binding buffer and bound proteins were eluted with 8M urea in binding buffer. The eluted samples were subjected to SDS-PAGE analysis and immunoblotting.

5.2.10 Protoplast preparation, Pulse-Chase labelling and immunoprecipitation

Protoplasts were prepared from axenic leaves (4 to 7 cm long) of *Nicotiana tabacum* cv. Petit Havana SR1. Leaves were cut and incubated overnight in the presence of an enzymatic mix containing 0.2% macerozyme and 0.4% cellulase prepared in K3 medium (Gamborg's B5 basal medium with minimal organics (Sigma), supplemented with 750 mg/l $CaCl_2$, 250 mg/l NH_4NO_3, 136.2 g/l sucrose, 250 mg/l xylose, 1 mg/l 6-benzylaminopurine, and 1 mg/l naphthalene-acetic acid, pH 5.5). Protoplasts were subjected to polyethylene glycol-mediated transfection as described by (Pedrazzini et al., 1997). Forty micrograms of plasmid were used to transform protoplasts at a concentration of 10^6 cells/ml. After transfection, protoplasts were allowed to recover overnight in the dark at 25°C in K3 medium (Pedrazzini et al., 1997) before pulse-chase experiments were performed. Pulse-chase labelling of protoplasts using Pro-Mix (a mixture of ^{35}S-Met and ^{35}S-Cys; Amersham Biosciences) was performed exactly as described in (Pedrazzini et al., 1997). Protoplasts were treated with 2 µM GA (Geldanamycin) at the beginning of the labelling period. After 2 hours incubation chase was

performed by adding unlabelled methionine and cysteine to a final concentration of 10 mM and 5 mM respectively. To recover protoplasts 3 volumes of W5 medium (9g/l NaCl, 0.37 g/l KCl, 18.37 g/l $CaCl_2$-$2H_2O$, 0.9 g/l glucose) were added, and protoplasts were pelleted by centrifugation at 100 x g for 5 min. Cells were frozen in liquid nitrogen and stored at -80°C. Homogenization of protoplasts was performed by adding 2 volumes of ice-cold homogenization buffer (150 mM Tris/HCl, 150 mM NaCl, 1.5 mM EDTA, and 1.5% (w/v) Triton X-100, pH 7.5) to the frozen samples supplemented with Protease inhibitor cocktail tablets. Immunoprecipitation of the GFP fusion proteins was performed as described (Pedrazzini et al., 1997), using rabbit polyclonal antisera raised against GFP. Immunoselected proteins were analyzed by SDS-PAGE and fluorography.

5.2.11 Affinity purification of antibodies

Toc64 antiserum was incubated for 90 min at RT with recombinant proteins covalently coupled to cyanogen bromide-activated Sepharose 4B (Amersham, Freiburg, Germany). The flow through was used as a specific epitope depleted serum. After washing the column (10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.3, 2.7 mM KCl, 140 mM NaCl), the bound antibodies were eluted with 0,2 M Glycine pH 2.6. The pH of the eluate was immediately adjusted with 1 M Tris-base pH 10 and used as a specific epitope serum. The activity of the purified antibodies was controlled by immunoblotting.

5.2.12 BN-PAGE

The buffers used for BN-PAGE and the gel itself were used according to Kikuchi and co-workers (Kikuchi et al., 2006). 1% dodecylmaltodise (Biomol, Hamburg, Germany) was used to solubilise total chloroplasts according to 100 µg chlorophyll content for 10 min on ice. Insoluble material was removed by centrifugation (256000 x g, 10 min, 4°C) and supplement with BN-PAGE sample buffer (Kikuchi et al., 2006). The sample was loaded onto a 1.5 mm thick 5-16% BN-PAGE and the run was performed as described (Kikuchi et al., 2006). For two-dimensional analysis the individual lines were excised from the first dimension, incubated for 30min at 20°C in 192 mM glycine, 25 mM Tris/HCl and 0.1% SDS. Subsequently, the gel lines were polymerised in the 0.75 mm SDS-PAGE stacking gel and the SDS-PAGE was run according to standard procedures.

5.2.13 Size-exclusion chromatogphy and glycerol gradient

Wgt ^{35}S-labelled pOE33 was preincubated as indicated and loaded onto a Superdex 200 (at 4°C) using 100 mM KoAC, 20 mM Hepes/KOH, pH7.6, 5 mM $MgCl_2$ as running buffer or a 4ml glycerol step gradient of 20 mM Tris/HCl pH7.6, 50 mM KoAc and 2 mM $MgCl_2$ containing 10%, 20% 30% 40% 50% and 60% glycerol. The gradient was fractionated after centrifugation (83 000 x g / 4°C / over night). Fractions were analysed by separation on SDS-PAGE and autoradiography.

5.2.14 Bioinformatic analysis

The topological models of Toc64 were assigned based on the database Aramemnon (Schwacke et al., 2003) using the protein from *P. sativum* (Q9MUK5), the protein from *A. thaliana* (At3g17970) and the protein from *Oryza sativa* (Os02g51810). Predictions were aligned and the averaged model was generated.

5.2.15 Calculation of the CI_{50} values for import inhibition and binding inhibition

For competition experiments a CI_{50} value was calculated according to the following assumptions: a K_m of 282 nM for preprotein translocation was observed previously (Dabney-Smith et al. 1999), and the concentration of precursor used here was way below. The import was performed for 10 min, which was in the linear range of the kinetic. All reactions were performed under inhibitor saturation. Here, the outer envelope proteins are about 2% of total chloroplasts (Joyard et a. 1991) with a concentration of Toc64 of 0.07 pmol per µg and Toc34 of 0.17 pmol per µg total outer envelope proteins (Vojta et al. 2004). Using 20 µg chlorophyll reflecting about 100 µg total proteins of chloroplasts leads to a final concentration of the receptors in the reaction volume in the nanomolar range, being at thousand fold below the final competitor concentration.

For binding experiments the determination of a correct CI_{50} value was not possible since neither kinetic measurements nor the K_m values was performed or determined for that interaction. Therefore, the values calculated are the concentrations where under similar experimental conditions 50% of the bound protein was eluted. The values are given for comparison.

6. Results

6.1 Toc64 topology

6.1.1 Toc64 contains a 30 kDa resistant fragment

Toc64 is a subunit of the chloroplast protein import machinery, which can be crosslinked to several subunits of the Toc complex (Sohrt and Soll, 2000). However, Toc64 was not co-purified with the Toc core components in linear sucrose gradient. Hence, a dynamic association of the Toc core translocon was suggested (Becker et al., 2004a). Primary sequence analysis of Toc64 revealed three motifs. The first motif exhibits homology to prokaryotic and eukaryotic amidases followed by the second motif the charged region and, the third C-terminal motif, the threefold repeated TPR-motif (Sohrt and Soll, 2000). Recent work suggests that Toc64 contains an N-terminal transmembrane region, which is essential and sufficient for targeting to chloroplasts (Lee et al., 2004). In order to perform functional studies of Toc64, the topological arrangement of the domains has to be elucidated. Therefore, accessibility of the protein to different proteases in the outer envelope membranes of pea chloroplasts was analyzed (5.2.4). Thermolysin is unable to cross the chloroplast outer membrane under conditions used (Cline et al., 1984); therefore, it will degrade only those portions of a protein that are exposed to the cytoplasm (Waegemann and Soll, 1992). Thermolysin treatment of right side out outer envelope vesicles (OEVs; Fig. 3A, lane 1) (5.2.4) revealed a 30 kDa protease resistant fragment (Fig. 3A, lane 2), which was not extractable by carbonate treatment (Fig. 3A, lane 4). Under the conditions used, Toc64 itself and a form with a truncated C-terminus (about 50kDa, Sohrt and Soll, 2000) were observed as well (Fig. 3A, lane 4). The 15 kDa proteolytic fragment previously identified as a TPR region (Sohrt and Soll, 2000) was detected in the supernatant after carbonate extraction of the membrane (Fig. 1A, lane 3). Confirming the previous observation, the carbonate resistant 30 kDa fragment was also obtained after thermolysin treatment of chloroplasts (Fig. 3A, lane 5). This fragment can be obtained independent of the protease used since incubation with proteinase K (Fig. 3B, lane 3) or chymotrypsin (Fig. 3B, lane 4) resulted also in a stable fragment of similar size. Interestingly, incubation of OEVs with chymotrypsin revealed an additional, slightly smaller carbonate resistant fragment (Fig. 3B, lane 4). To assay whether the resistant fragment exhibits the properties of a membrane protected region, the results were compared to the proteolytic digestion of Toc159, since it was demonstrated that Toc159 contains a 52 kDa C-terminal domain, which is protected by the membrane (Becker et al., 2004b). Similar protease treatment of OEVs as performed for the analysis of Toc64 results in appearance of the protease resistant membrane protected 52 kDa fragment of Toc159 (Fig. 3B, lane 6-8).

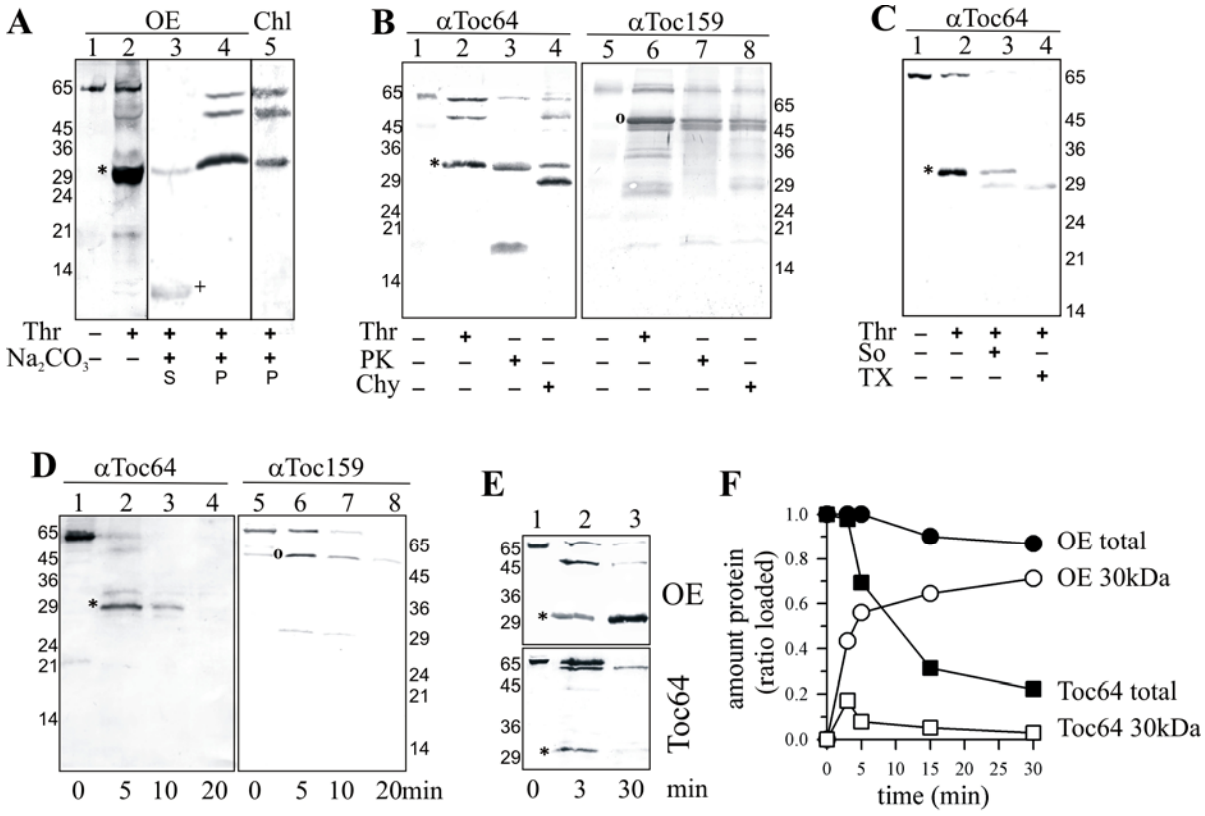

Figure 3. Toc64 contains a 30 kDa resistant fragment.

A) 10 µg OEVs (OE, lane 1-4) or chloroplasts (500 µg chlorophyll, Chl, lane 5) were incubated with thermolysin (Thr) (1µg for OE, 25 µg for chloroplasts, lane 2-5) followed by carbonate extraction (Na_2CO_3) (lane 3-5), untreated OEVs (10%, Lane 1). Proteins of the supernatant (S, lane 3) or the pellet (P, lane 4, 5) were separated by SDS-PAGE and immunodecorated with αToc64 antiserum. **(B)** 10 µg OEVs were incubated with different proteases. 5 µg thermolysin for 20 minutes on ice (Thr, lane 2, 6), 40 mg/ml proteinase K for 2 minutes on ice (PK, lane 3, 7) or 1 µg chymotrypsin for 5 minutes at RT (Chy, lane 4, 8), Untreated OEVs (lane 1, 5). Products were analyzed by SDS-PAGE and immunodecoration with αToc64 (lane 1-5) or αToc159 antiserum (lane 6-10). **(C)** 10 µg OEVs were incubated with thermolysin (Thr, lane 2-4) either while sonication (So, lane 3) or in the presence of Trition X-100 (TX, lane 4), untreated OEVs (lane 1)**.** Proteins were separated and incubated with αToc64 antiserum. **(D)** 10 µg OEVs were incubated with 1 µg trypsin on ice for the indicated time period and separated by SDS-PAGE and immunodecorated with Toc64 (lane 1-4) or Toc159 antibodies (lane 5-8). **(E)** 50 µg OEVs supplemented with 1 µg BSA (OE) or 1 µg expressed Toc64 suplemented with 40 µg IEVs (Toc64) were incubated with 25 µg thermolysin for the indicated time. Proteins were separated and detected with αToc64 antiserum (lane 1-5). As control, 50% of overexpressed Toc64 or OE is shown (lane 1). For (A-E) "*" indicates the 30 kDa fragment of Toc64, "+" the 15 kDa fragment of Toc64 and "**o**" the 52 kDa fragment of Toc159. **(F)** Proteolytic digest of outer envelope (circle) or expressed Toc64 (square) was performed as in (E) for the indicated time. The amount of total protein (OE or Toc64 total, closed symbol) or the 30 kDa fragment (Toc64 30kDa, open symbol) in each time was determined by densitometry and expressed as ratio to loaded protein.

Both, the stability of the 30 kDa fragment of Toc64 and the occurrence of the 52 kDa fragment of Toc159 implies an intermembrane space localization of the 30 kDa fragment.

To further test if the 30 kDa fragment indeed constitutes an intermembrane space localized domain, OEVs were incubated with thermolysin either during sonication (Fig. 3C, lane 3) or in the presence of Triton X-100 (Fig. 3C, line 4). Both treatments are thought to disrupt the membrane (Becker et al., 2004a). Indeed, this treatment resulted in the degradation of the 30 kDa fragment of Toc64 (Fig. 3C, lane 3, 4). Furthermore, upon treatment of OEVs with trypsin which, in contrast to thermolysin, penetrates the membrane more rapidly as determined by the degradation of the 52 kDa fragment of Toc159 (Fig. 3D, lane 8), the 30 kDa fragment was degraded at a similar rate as the 52 kDa fragment of Toc159 (Fig. 3D, compare lane 2-4 and 6-8).

Tom70 of the mitochondrial outer membrane import apparatus shares several features with Toc64; it exposes a TPR domain towards the cytosol and contains a proteolytically stable core domain, which has a specific binding site for preproteins (Brix et al., 2000). To exclude the possibility of protease resistance of Toc64 being caused by a specific fold of the domain, soluble expressed Toc64, or OEVs were incubated with thermolysin for varying lengths of time (Fig. 3E). The secondary structure content of the expressed Toc64 was confirmed by CD-spectroscopy (Becker, personal communication) and light scattering (Tews, personal comunication). The protein and lipid content was adjusted by supplementing Toc64 with inner envelope vesicles and OEVs with BSA. Subsequent proteolysis revealed that the 30 kDa fragment of Toc64 present in the outer envelope remained thermolysin resistant even after 30 minutes (Fig. 3E, lane 3, OE). Here the amount of the detectable protein was only slighly reduced (Fig. 3F, closed circle). The fraction represented by the 30 kDa fragment, however, increased over the time period of the proteolysis (Fig. 3F, open circle). In contrast, the proteolytic fragment of the expressed Toc64 with similar migration properties as the 30 kDa fragment of the membrane inserted Toc64, which initialy appered (Fig. 3E, lane 2, Toc64) was degraded after 30 minutes (Fig. 3E, lane 3, Toc64). In contrast to the 30 kDa fragment of the membrane inserted protein, which was enhanced in the same manner as the protein was degraded (Fig. 3F, circle), the 30 kDa fragment of the soluble protein disappeared over time (Fig. 3F, square). All results together suggest that the TPR containing domain of Toc64 is exposed to the cytosol (Sohrt and Soll, 2000), whereas a second domain of about 30 kDa is protected by the chloroplast outer envelope.

6.1.2 Both, the amidase and the charged domain contribute to the formation of the protease resistant 30 kDa fragment

To identify the domain which represents the stable 30 kDa fragment, Toc64 antibodies were purified against two different epitopes representing different domains of Toc64, GST-Ami and GST-Cha (5.1.3, Fig. 2A). The efficiency of the purification and the activity of the remaining antibodies was confirmed by immunoblotting using expressed polypeptides (5.2.6) representing the amidase domain (Fig. 4A, lane 1), the charged domain (Fig. 4A, lane 2) or the TPR domain (Fig. 4A, lane 3). Thermolysin treated OEVs (5.2.4) were subsequently immunodecorated using these antibodies (Fig. 4B, lane 2, 4, 6). Here both antibodies recognizing the amidase and the chareged region decorated the 30 kDa fragment (Fig. 4B, lane 4, 6). It was demonstrated that the purified antibodies against TPR region do not recognize the 30 kDa fragment (Sohrt and Soll, 2000). From these results it can be concluded that both, the amidase and the charged domain contribute to the 30 kDa fragment.

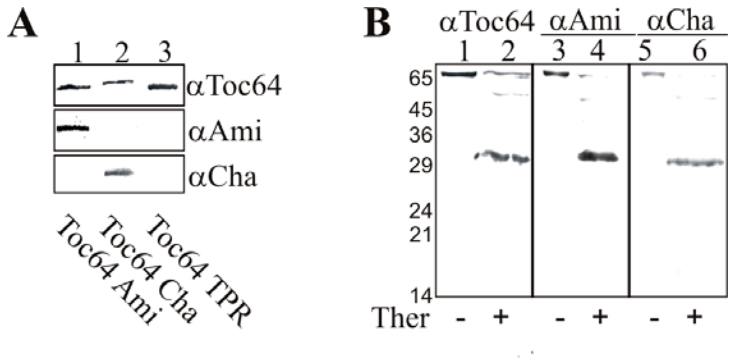

Figure 4. Recognition of the 30 kDa fragment by domain specific antibodies.
(A) expressed Toc64Cha (lane 1, see Fig. 1A), Toc64TPR (lane 2) or Toc64Ami (lane 3) were separated by SDS-PAGE and detected with αToc64 or with the purified antibodies against Toc64Ami (αAmi) or Toc64Cha (αCha). **(B)** OEVs were incubated with thermolysin (lane 2, 4, 6), untreated OEVs (lane 1, 3, 5). Proteins were separated and detected with the indicated antiserum.

6.1.3 Topological modeling of Toc64

So far it was demonstrated that the 30 kDa protease resistant fragment of the protein present in the outer envelope is at least partially represented by the amidase and charged regions. To date, the topology of the protein was based on predictions (Sohrt and Soll, 2000) and on proteolysis of the proteins from *P. sativum* (Sohrt and Soll, 2000) or *P. patens* (Rosenbaum and Theg, 2005) after import into the outer envelope of chloroplasts from *P. sativum*. It was documented that the N-terminal transmembrane region has an N_{in}-C_{out} orientation (Lee et al., 2004). To explain these results a topological model of Toc64 was created (5.2.14) (Fig. 5). However, topology prediction by programs has to be taken with care since the prediction

quality of the programs should not be overestimated (Möller et al., 2001). Therefore, three Toc64 proteins - from *P. sativum*, *A. thaliana* and *O. sativa* - were analysed regarding their predicted transmembrane regions by all of the programs (Fig. 5). The first transmembrane region, which has been experimentally confirmed by (Lee et al., 2004), was not assigned for the *A. thaliana* protein (Fig. 5). However, the alignment of all three topological models considering all predicted transmembrane domains by individual programs indicates the presence of two further transmembrane spanning regions.

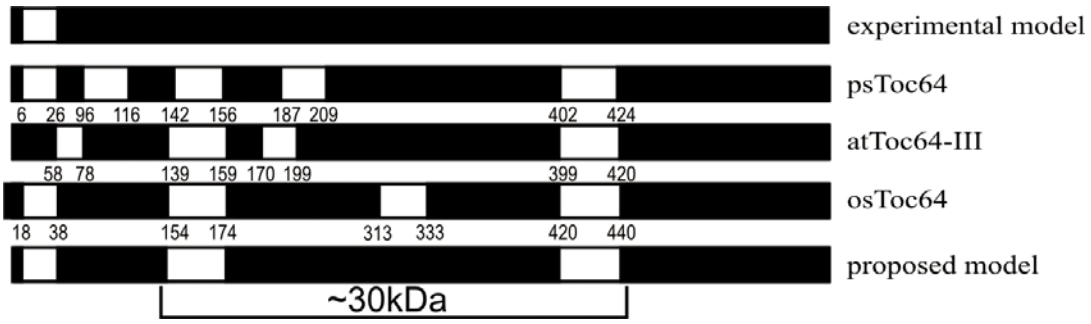

Figure 5. The topological model of Toc64.
The topological model of Toc64 was investigated as described in (4.2.14). Shown are the experimental model with the confirmed first transmembrane domain (bar 1), all predicted transmembrane domains for the proteins from *P. sativum* (bar 2), *A. thaliana* (bar 3) and *O. sativa* (bar 4). Bar 5 indicates the proposed consensus model for Toc64 topology. Bars are aligned according to sequence alignment. Transmembrane domain: (TM = white).

To confirm this model, Toc64 itself and truncated constructs containing deletions of the three main domains of Toc64 (5.1.3, Fig. 2B) were imported into chloroplasts (5.2.2). For that, the chloroplast localized *A. thaliana* protein Toc64-III (Chew et al., 2004) was used, which has a similar amino acid composition of the N-terminal domain, proposed to function as insertion signal (Lee et al., 2004), as Toc64 from pea (Fig. 6). The Arabidopsis Toc64-III protein displays a high amino acid sequence identity (67%) with pea Toc64 (Fig. 6). The polypeptides were synthesized by *in vitro* transcription/translation in the presence of S^{35}-methionine (5.2.1) and incubated with isolated chloroplasts (5.2.3). Subsequently, the topology of the inserted proteins was analyzed (Fig. 7). As a control for import efficiency, pSSU was imported into chloroplasts in parallel (Fig. 2A, lane 2). Protease treatment after completion of the import reaction of Toc64 yielded two prominent proteolytic fragments (Fig. 7A, lane 4). The larger fragment with an apparent molecular weight of about 30 kDa was resistant to carbonate extraction indicating its membrane insertion (Fig. 7A, lane 5). In contrast, the smaller fragment was largely extractable. Toc64 lacking the TPR domain (Fig. 7A, lane 6) revealed the same insertion and proteolytic behavior as observed for the full length protein (Fig. 7A,

lane 7, 8). This is consistent with the observation that the TPR region is exposed on the cytosol (Fig. 3).

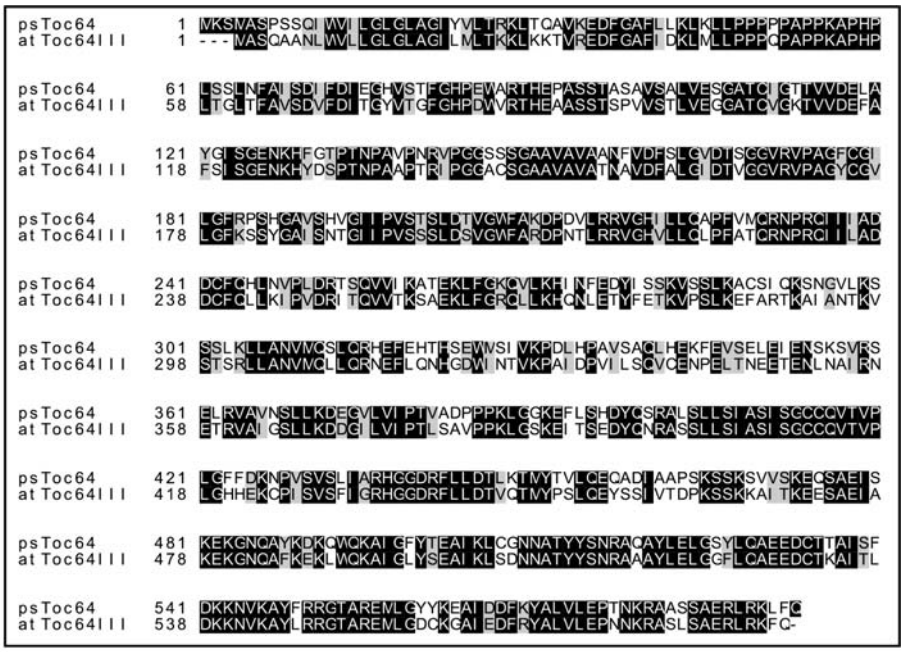

Figure 6. Sequence alignment of pea Toc64 with the homologous protein atToc64III in Arabidopsis.
The *Arabidopsis* protein is designated according to the location of the gene on the chromosome. ps: *P. sativum*, at: *A. thaliana*. Identical amino acids are shown in black box and homologous ones in grey box.

Deletion of the amidase and the charged domain resulted in a weak association of the protein with the membrane surface (Fig. 7B, lane 5, 6). However, as for the Toc64 lacking the N-terminal region, no insertion of Toc64ΔAC could be observed as determined by carbonate extraction (Fig. 7B, lane 5, 6). This suggests further transmembrane regions within the deleted region. Deleting the amidase and the charged region individually (Fig. 7B) resulted in insertion of the proteins into the membrane (Fig. 7B, lane 2, 4). However, the topology of the protein could not be restored by either one of the deletion constructs since no protease resistant fragment could be obtained (Fig. 7A, lane 10, 12). This observation confirms the presence of the transmembrane regions within the amidase and the charged region, which is disrupted in both constructs (Model Fig. 5). It further suggests that all transmembrane regions are required for correct topology of the protein. To identify the smallest region representing the protease resistant fragment, constructs (Δ1, Δ2) with C-terminal truncations were generated (5.1.3, Fig. 2B). Again, importing the construct bearing the 437 N-terminal amino acids (Δ1) revealed the same 30 kDa fragment after proteolysis as for Toc64ΔTPR (ΔTPR) (Fig. 7C, lane 2, 4). Using only the first 250 amino acids (Δ2) of atToc64 the protein was still anchored to the membrane (Fig 7C, lane 2) confirming the presence of the localization signal

in the N-terminus, but no fragment was observed after protease treatment (not shown). This observation is the result of the false topology of the protein after insertion. In conclusion, the results from the experiments presented in Figure 7 suggest that the protease resistant region has to be located between amino acids1 and 437.

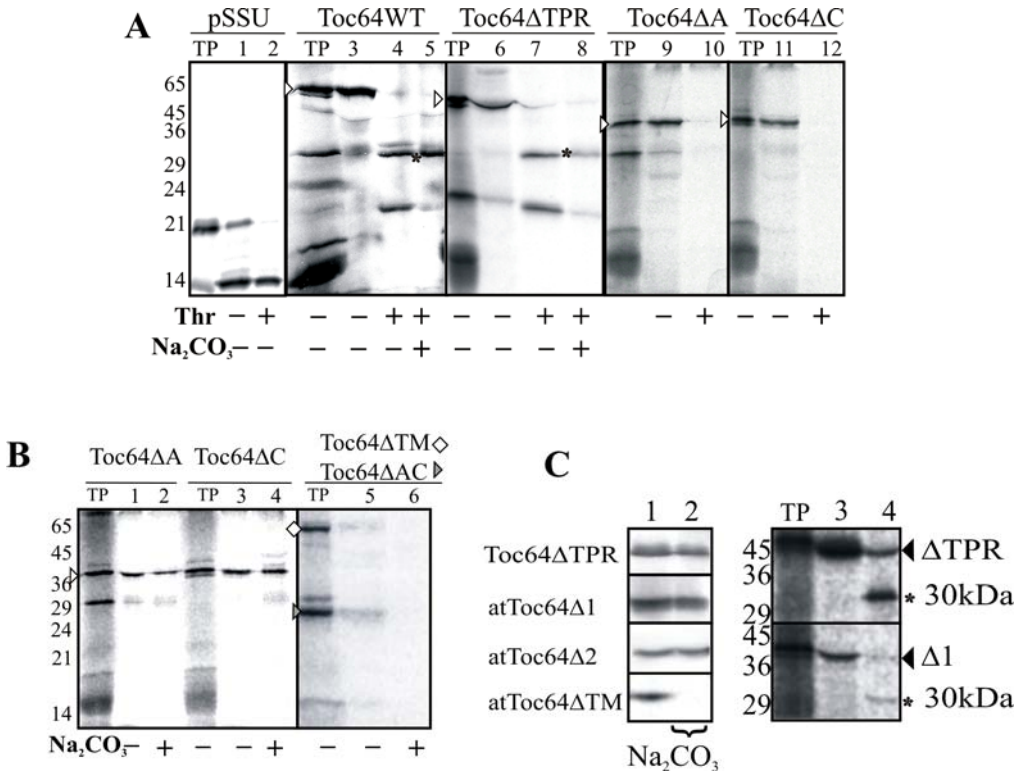

Figure 7. Import of Toc64 and its constructs into isolated chloroplasts.
(A) Rlt-^{35}S-labelled pSSU, Toc64wt, Toc64ΔTPR, Toc64ΔA or Toc64ΔC were imported into isolated chloroplasts (10 µg chlorophyll). Re-isolated chloroplasts were either not treated (lane 1, 3, 6, 9, 11) or treated with thermolysin (3 µg/5µg chlorophyll,Thr, lane 2, 4, 5, 7, 8,10, 12) followed by carbonate treatment for Toc64wt and Toc64ΔTPR (Na_2CO_3, lane 5, 8). TP: 10% translation product (indicated by white triangle). **(B)** Rlt-^{35}S-labelled Toc64ΔA, Toc64ΔC, Toc64ΔAC or Toc64ΔTM were incubated with chloroplasts as in (A). Re-isolated chloroplasts were either not treated (lane 1, 3, 5; 15 minutes, 25^{o}C) or treated with carbonate (Na_2CO_3, lane 2, 4, 6). **(C)** Rlt-^{35}S-labelled Toc64ΔTPR, Toc64Δ1 (aa1-437), Toc64Δ2 (aa1-250) or Toc64ΔTM were imported as in (A). Re-isolated chloroplasts were either not treated (lane 1, 3) or treated with carbonate (lane 2). For Toc64ΔTPR (Δ) and Toc64Δ1 (Δ1) constructs after import re-isolated chloroplasts are incubated with thermolysin (lane 4). For all experiments chloroplast proteins were separated by SDS-PAGE and visualized by phosphor-imager exposure. The 30 kDa protease resistant fragment is indicated by a star. TP, 10% translation product

6.1.4 The 30 kDa fragment is membrane protected

So far the data indicated that the membrane inserted Toc64 has a stable domain of 30 kDa between amino acid 1 and amino acid 437. To further confirm the membrane protected region a pegylation assay was used (5.2.5) (Lu and Deutsch, 2001). After import of proteins into chloroplasts, samples were incubated with pegylated maleimide (PEG-MAL), which modifies the cysteine residues. PEG-MAL reagent does not cross membranes even upon incubation at 4°C for 24 h at high concentration (Lu and Deutsch 2001) resulting in crosslinks only on cytoplasmic exposed cysteines. The apparent molecular weight of the protein is shifted by about 10 kDa after crosslinking of a single PEG-MAL molecule to a cysteine within the protein when analyzed by SDS-PAGE. First to test whether PEG-MAL penetrates the membrane under conditions used here, the precursor form of Tic22, the intermembrane space localized component of the translocation machinery, was imported into chloroplasts (5.2.3) (Fig. 8B). Tic22 contains two cysteines, which are accessible for PEG-MAL as judged from incubation of the translation product only (Fig. 8B, TP). After import into the intermembrane space of the chloroplast Tic22 was processed and occurred as mature form (Fig. 8B, lane 1). To assay whether imported Tic22 can be modified by PEG-MAL, the precursor form not inserted into chloroplasts was removed by thermolysin before addition of PEG-MAL (Fig. 8B, lane 2). However, no labeling of the imported Tic22 was observed (Fig. 8B, lane 2) except when the membrane was disrupted by addition of SDS (Fig 8B, lane 3). In the latter case two modifications were observed indicating that the mature form of Tic22 is generally accessible to PEG-MAL modification. This confirmed that PEG-MAL did not pass the outer membrane under the conditions used. Accordingly, protection of the resistant domain of Toc64 to proteolysis by the membrane could be probed. Since the cysteine distribution of Toc64 from pea and from *A. thaliana* is slightly different (Fig. 8A), both proteins were imported into chloroplasts (Fig. 8C, lane 1, 2) and subsequently labelled with PEG-MAL (Fig. 8C, lane 3, 4). For both proteins only two shifted bands were observed (Fig. 3B, lane 3, 4), even though all cysteines were accessible before insertion in translation product (Fig. 8C, TP). When probes after pegylation were treated with proteases the 30 kDa fragment was observed (Fig. 4C, lane 5, 6). This observation suggests that the modified cysteines are localized within regions distinct from the 30 kDa membrane protected domain. (Fig. 8C, lane 5, 6). However, one intermediate of about 40 kDa was obtained for the protein from pea suggesting that the crosslink site might be close to the cleavage site of the protease and therefore somehow less accessible (Fig. 8C, lane 6). To confirm that the 30 kDa fragment was not labeled in this case, samples were first protease treated after import into chloroplasts (Fig. 8C, lane 7, 8) and

subsequently incubated with PEG-MAL (Fig. 8C, lane 9, 10). In this case no shift was observed suggesting that no cytsein was freely exposed to the chloroplast surface. To control whether cysteines are protected by the membrane, the pegylation of the soluble expressed Toc64 from *P. sativum* was tested. The results were compared to the observations for the imported Toc64 from *P. sativum* (Fig. 8D).

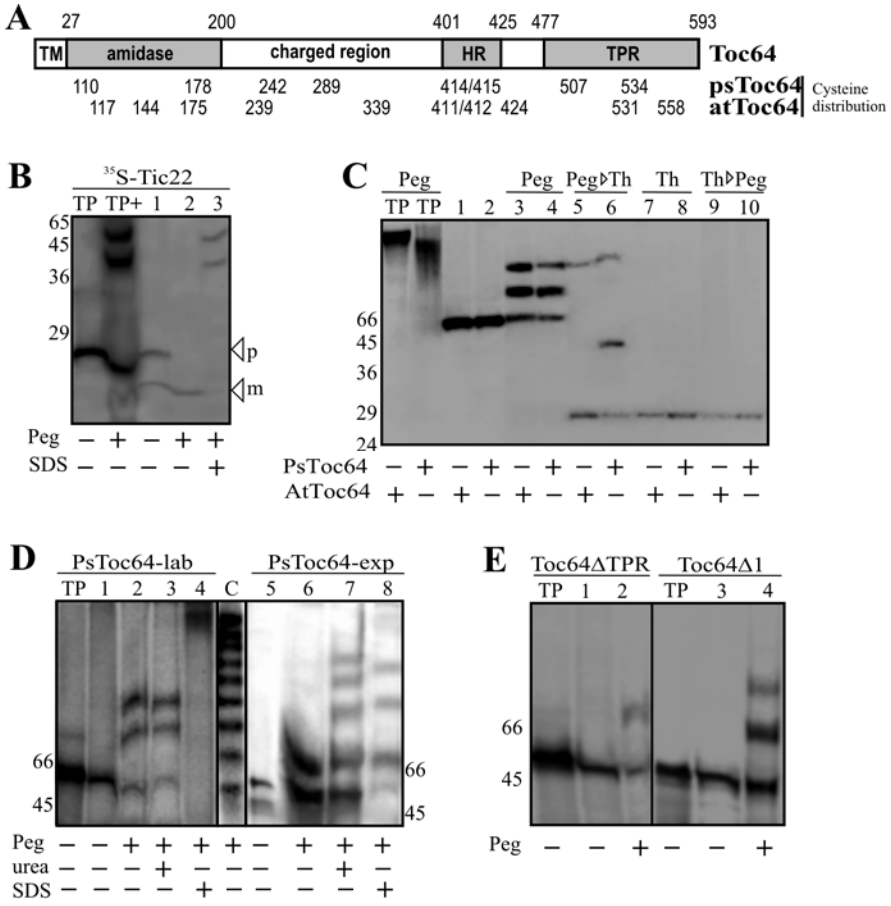

Figure 8. Localization of the protease resistant fragment.
(A) Shown is the domain structure of Toc64 and the cysteine distribution within atToc64 from *A. thaliana* and psToc64 from *P. sativum*. **(B)** Radioactive labeled Tic22 (TP) was incubated with PEG-MAL (lane TP+) or imported into chloroplasts as in Figure (7A). Re-isolated chloroplasts were either not incubated (lane 1) or incubation with PEG-MAL (Peg, lane 2, 3) and followed by solubilization 0.1% SDS (lane 3). P: precursor, m: mature protein. **(C)** Radioactive labeled atToc64 or psToc64 was imported into chloroplasts. Re-isolated chloroplasts were either not incubated (lane 1, 2) or incubated with PEG-MAL (Peg, lane 3-6)) followed by thermolysin digestion (Peg>Thr, lane 5, 6), or re-isolated chloroplasts were incubated first with thermolysin (Thr, lane 7, 8) followed by incubation with PEG-MAL (Thr>Peg, lane 9, 10). For control, translation product was incubated with PEG-MAL for the same time period (TP). **(D)** Radioactive labeled psToc64 (Lab) (TP, 10% for control) was imported. Reisolated chlorplasts were either not treated (lane 1) or treated with PEG-MAL (lane 2-4) in the presence of either 2M urea (lane 3) or 0.1% SDS (lane 4). Soluble expressed psToc64 (exp) (lane 5-8) was incubated with PEG-MAL (lane 6-8) either in the presence of 2M urea (lane 7) or 0.1% SDS (lane 8). Lane C (C) shows the translation product incubated with PEG-MAL for 5 min to compare the pattern expected. **(E)** Radioactive labeled atToc64ΔTPR or atToc64Δ1 (TP, 10%) were imported. Re-isolated chloroplasts were either not incubated (lane 1, 3) or incubated with PEG-MAL (lane 2, 4).

After pegylation, the expressed protein was labelled at one site after identical incubation times (Fig. 8D, lane 6). After incubation with 2M Urea, the imported protein was still labeled at two cysteines (Fig. 8D, lane 3). In contrast, the expressed protein shows a labeling of at least 6 cysteines (Fig. 8D, lane 7). A similar result was obtained when the expressed protein was incubated with 0.1% SDS (Fig. 8D, lane 8). However, the 0.1% SDS disrupted the chloroplast membrane and subsequently the imported protein became hyper-modified (Fig. 8D, lane 4). These results suggest that many of the cysteins were protected by the membrane. To define the number of PEG-MAL molecules crosslinked, migration of the imported Toc64 after crosslinking with PEG-MAL without further treatments (Fig. 8D, lane 2) was analyzed by SDS-PAGE. Comparing the migration of the crosslink products of psToc64 after import into chloroplasts (Fig. 8D, line 2) with those observed after a short treatment of the translation product (Fig. 8D, line C), crosslinking of two and three cysteins was confirmed (Fig. 8D, compare lane 2 and C).

To identify the localisation of the cysteine crosslinks, the pegylation after import in chloroplast of atToc64 from *A. thaliana* lacking the TPR motif (Toc64ΔTPR) or ending at the position 437 (Toc64Δ1) was compared. As expected, Toc64ΔTPR was labeled at one position (Fig. 8E, lane 2). Considering the proposed topological model (Fig 5) and that the 30 kDa fragment is localized between amino acid 1 and 437 (Fig.7), one could consider amino acid 117, 144 or 424 to be modified in Toc64ΔTPR. However, these cysteines are not present in the protein from *P. sativum* (Fig 8A) and therefore this assumption would contradict the obtained similar labeling of the full length proteins from *P. sativum* and *A. thaliana* (Fig. 8C). Following this argumentation, the labeling of the cysteine 117 which corresponds to the cysteine at position 110 the protein from *P. sativum* could be proposed. To confirm the observation, the construct ending at position 437 (Toc64Δ1) was imported and labeled. Interestingly, this protein was modified at two cysteine residues (Fig. 4E, lane 4). This result can be interpreted by proposing that either cysteine 144 or cysteine 424 became accessible by removal of the region starting at amino acid 437 of Toc64. Hence, these results demonstrate that most of the cysteines are protected by the membrane since only three were accessible from the cytsolic side. Summarizing, a 30 kDa protease resistant domain was observed, which can not be targeted from the outside but from the inside by PEG-MAL. For both proteins from *P. sativum* and *A. thaliana* three exposed cysteines are present. As will be discussed later, it could be suggested that cysteines 110/507/534 of pea and 117/531/558 of *A. thaliana* protein are the exposed amino acids. Considering all observations, the 30 kDa region is localized between amino acid 110 and 507 and exposed to the intermembrane space.

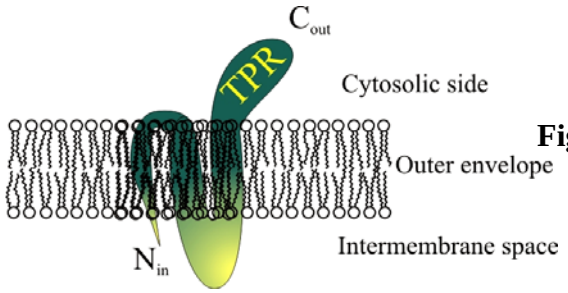

Fig. 8F. Topological model of Toc64.
See text for detailed explanation.

All results presented in the previous sections are summarized in the presented model (Fig. 8F). The TPR containing domain of Toc64 is cytosolic exposed (Sohrt and Soll, 2000), whereas a second domain of about 30 kDa is exposed to the intermembrane space and protected by the chloroplast outer envelope. The protein has a N_{in}-C_{out} orientation (Lee et al., 2004). The topological model (Fig. 5) containing three transmembrane domains is well supported by the obtained results (Fig. 8F).

6.2 Toc64 association with the complex

6.2.1 The intermembrane domain of Toc 64 is a part of the translocon

After establishing the existence of an intermembrane domain, its interaction with other components of the outer envelope was analysed. It can be speculated that the intermembrane space domain of Toc64 is involved in the formation of an intermembrane space complex, which involves Toc64, Toc12 and an Hsp70 isoform (Becker et al., 2004b). However, Toc64 can be crosslinked to several subunits of the Toc complex (Sohrt and Soll, 2000) and it was not co-purified with the Toc core components in linear sucrose gradient. Hence, a dynamic association of the Toc core translocon was suggested (Schleiff et al., 2003a) and a GTP dependent association was shown (Becker, 2005). To further explore the complex, chloroplasts were solubilised and subjected to BN-PAGE (Fig. 9A, B). In BN-PAGE protein complexes are separated without denaturation. Immunodecoration of the first dimension with antiserum against Toc64 revealed a complex of about 700 kDa (not shown). After immunodecoration of the second dimension, it was confirmed that this complex contains Toc159, Toc75, Toc34, Toc64 Toc12 and even Hsp70 (Fig. 9A, black arrow). A further complex could be obtained at about 550 kDa (Fig. 9A, grey arrow) containing the 86 kDa fragment of Toc159, Toc75, Toc34, Toc64 and Toc12. At the same molecular weight, no co-migration of the outer envelope protein Oep16 or the Tic components Tic40 or Tic110 was observed. Therefore, two different forms of the Toc complex were obtained. Interestingly,

both complexes contained Toc64. The difference to previous results can be explained by the use of different solubilisation and purification strategy. That alteration of the isolation strategies can reveal the purification of different complexes, however, was previously found for other translocation machineries like the Tom complex in the outer mitochondrial membrane (e.g. Künkele et al., 1998; Athing et al., 1999).

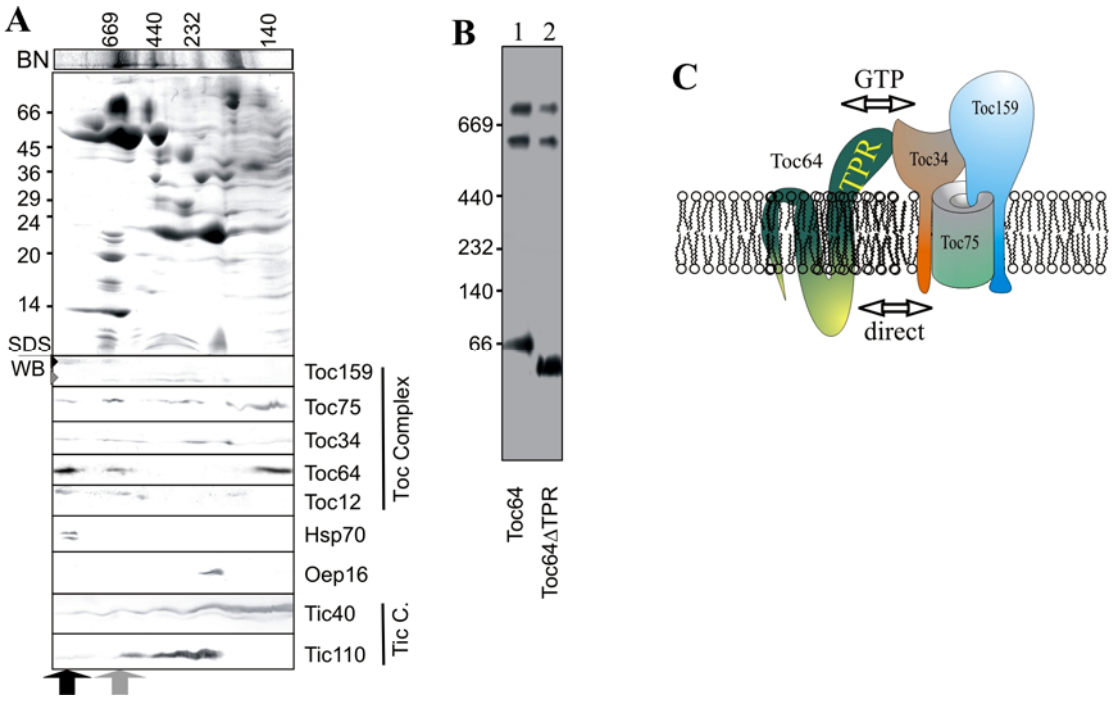

Figure 9. The translocon of the outer envelope.
(A, B) Chloroplasts (100 µg chlorophyll) were solubilized by addition of 1.5% dodecylmaltoside and subjected to BN-PAGE (BN). One lane was excised from the gel and proteins were separated in the second dimension on SDS-PAGE (SDS) followed by transfer to nitrocellulose membrane and detection with the indicated antibodies (WB). The arrows indicate the migration of the Toc components. In **B**, radioactive labeled Toc64 (lane 1) or Toc64ΔTPR (lane 2) were incubated with chloroplasts prior to solubilization. The first dimension was analyzed by autoradiography. **(C)** A model of GTP-dependent association of Toc64 to the Toc translocon is presented.

To understand, whether the intermembrane space part of the Toc64 also assembles with the complex, Toc64 and Toc64ΔTPR were imported into chloroplasts prior to solubilisation (Fig. 9B). Both proteins assembled into complexes of about 700 and 550 kDa. Therefore, the TPR domain is not required for the assembly of Toc64 into the complex. However, a size difference between complexes containing Toc64 with or without TPR domain was not observed. This can be explained by the limited resolution of the BN-PAGE and by a high ratio of endogenous Toc64 to imported Toc64. In line with this, fractionation of complexes of solubilised chloroplasts by size exclusion chromatography revealed the previously observed

Toc complex of about 550 kDa (Becker personal communication). Further, after thermolysin treatment of chloroplasts before solubilisation, the Toc complex migrates with a molecular weight of about 300-350 kDa and interestingly, the 30 kDa fragment of Toc64 migrates with the Toc complex, suggesting a second association site within the Toc64 beside the identified interaction side within the TPR domain (Becker, personal comunication).

The translocon at the outer envelope of chloroplasts contains at least three proteins discussed as receptors for chloroplastic precursor proteins, namely Toc34, Toc64 (Soll and Schleiff, 2004) and Toc159 (Becker et al., 2004b). It was demonstrated that Toc34GMP-PNP is the docking site for the dynamically associated Toc64 in the Toc core complex and this interaction is mediated by the cytosolic exposed TPR domain of Toc64 (Becker, 2005). The interaction between the Toc core components and Toc64 is specific. Since, Toc64TPR interacts with the cytosolic domain of Toc34 in a GTP dependent manner (Becker, 2005) (Fig. 9C, Model). It can be concluded that the intermembrane space is involved in the formation of Toc complex and on the cytosolic phase of the membrane the association of Toc64 via its TPR with the Toc core complex is modulated by GTP (Fig. 9C, Model).

6.3 Toc64 is a preprotein receptor

6.3.1 Interaction of precursor proteins with Toc64

After demonstrating that Toc64 is a part of the translocon, the function of Toc64 in preprotein translocation into chloroplast has to be elucidated. Therefore the soluble expressed proteins Toc64, Toc34ΔTM and cBag (Bcl2-associated anthanogene) were used to inhibit the import of prepoteins into chloroplasts (Fig.2A, Fig 10A). The correct folding of the expressed proteins was confirmed by structural analysis (Becker and Oreb, personal communication). The translocation of wheat germ translated (wgt) ^{35}S-labelled precursor of the thylakoid lumen localised oxygen evolving complex subunit of 33 kDa (wgt-pOE33) containing a bipartite targeting signal was reduced by more than 80% of the control in the presence of 10 µM Toc64 (Fig 10A, 3-6; Fig 10B, black triangle) but significantly less in the presence of 10 µM Toc34ΔTM$_{GTP}$ from *P. sativum* (Toc34) (Fig. 10A, 3-6; Fig 10B, white triangle), which was previousely established as a preprotein receptor (Sveshnikova et al., 2000). No inhibition effect was observed for cBag (Bcl2-associated anthanogene). cBag was found to inhibit the Hsp70 dependent translocation into mitochondria (Young et al. 2003). This finding indicates that Hsp70 might not be essential for targeting of pOE33. The influence of the Toc proteins used is not restricted to wgt-pOE33 since the translocation of the wheat germ translated ^{35}S-labelled precursor form of the small subunit of RubisCO (wgt-pSSU) into

isolated organelles was also reduced by 80% in the presence of 10 µM of Toc64 (Fig 10B, black circle) or of Toc34ΔTM$_{GTP}$ (Fig 10B, white circle).

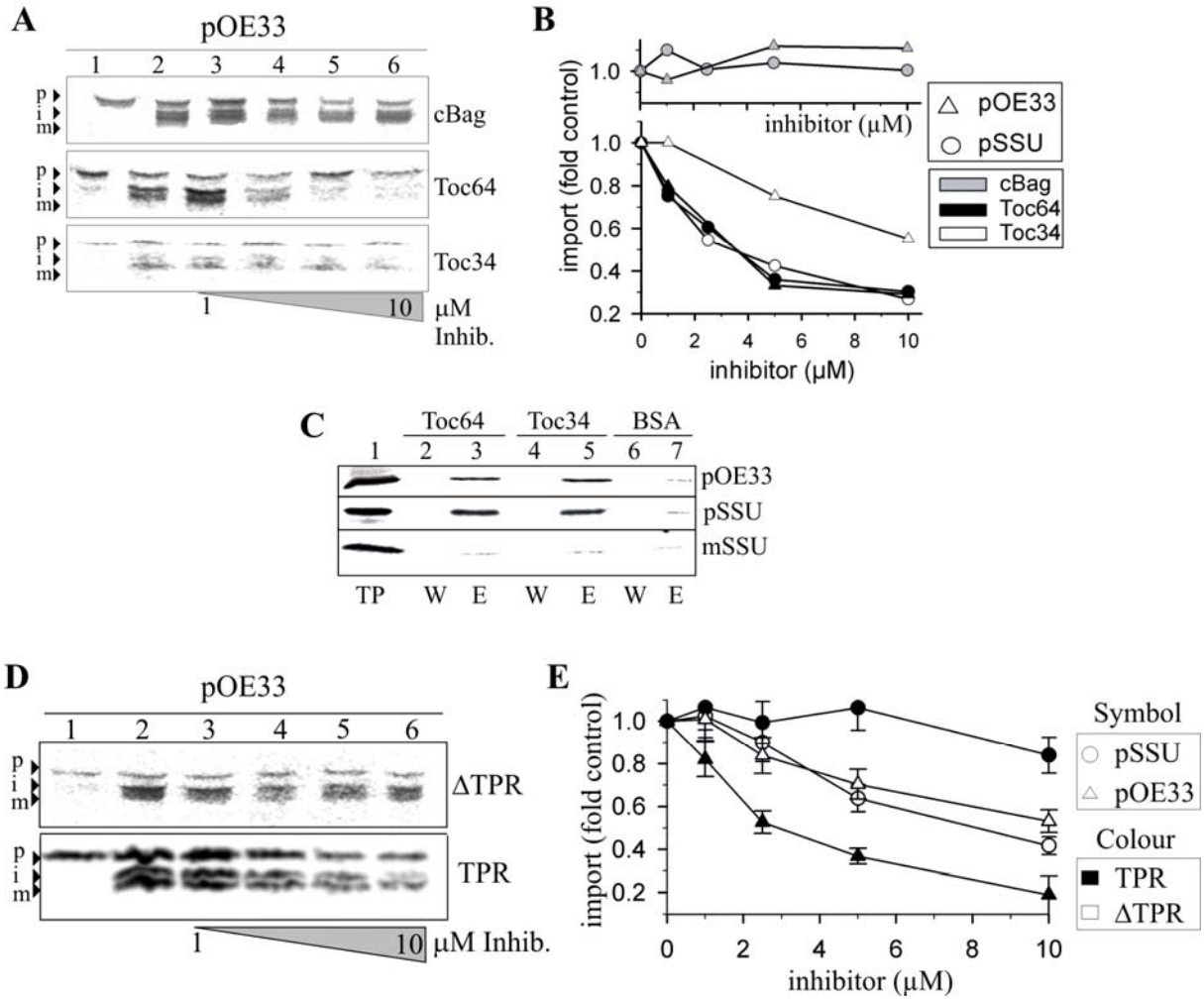

Figure 10. Toc64 is a preprotein receptor.
(A, B) Chloroplasts (20 µg chlorophyll) were incubated with wgt S^{35}-labelled pOE33 (A, B triangle) or pSSU (B circle) in the presence of increasing amounts 1-10 µM (final concentration, lane 3-6) of cBAG (A, upper part; B, grey), Toc64 (middle part, black) or Toc34ΔTM (lower part, white) at 25°C (lane 2-6) for 10 min. A control was kept at 4°C (lane 1). Chloroplasts were reisolated and import was visualized, quantified and compared to import without competitor (line 2). The average of at least 3 independent experiments is shown. Error bars are omitted for clarity. (p, precursor; i, stromal intermediate; m, mature protein) **(C)** Wgt S^{35}-labelled pOE33, pSSU or mSSU were incubated with a Toc64 (lane 2-3), Toc34 (lane 4-5) or BSA (lane 6-7) affinity matrix. Proteins in the final wash (W) and elution (E) are visualized. Lane 1 (TP) shows 20% of the protein loaded. **(D, E)** Chloroplasts (20 µg chlorophyll) were incubated with wgt S^{35}-labelled pOE33 (D; E, triangle) or pSSU (E, circle) in the presence of 1-10 µM (final concentration, lane 3-6) Toc64ΔTPR (D, upper part; E, white) or Toc64TPR (D, lower part; black) at 25°C (lane 2-5) for 10 min. A control was kept at 4°C (lane 1). Chloroplasts were reisolated and subjected to SDS-PAGE and import was visualized by autoradiography. (p, precursor; i, stromal intermediate; m, mature protein). Translocation efficiency was quantified and compared to the import without competitor (E). Average of 3 independent experiments is shown. Inhib: inhibitor

Furthermore, the direct association of the precursor proteins pSSU and pOE33 to Toc64 and to Toc34 as control was tested. Both wgt preproteins bind to a matrix coated with either receptor (Fig. 10C, lane 3, 5), but only with background levels to a BSA coated matrix (Fig. 10C, lane 7). The recognition of preproteins is transit sequence dependent since no interaction of the mature form of SSU with either Toc34 or Toc64 above background level was observed (Fig. 10C, lane 3, 5). This result demonstrates that Toc64 acts as a receptor for both tested preproteins.

6.3.2 The different domains of Toc64 facilitate recognition of preprotein

To identify the domains of Toc64 acting as a receptor for preproteins, two truncated constructs Toc64ΔTPR and Toc64TPR (5.1.3, Fig. 2A), were used to inhibit the import of wgt-pSSU, and wgt-pOE33 into chloroplast (5.2.3) (Fig. 10D, E). Toc64TPR inhibited the translocation of wgt-pOE33 into chloroplasts (Figure 10D, TPR, lane 3-6; 10E, black triangle) with the same efficiency as full-length Toc64 (Fig. 10A). In contrast, the import of reticulocyte lysate translated pOE33 was not affected by Toc64TPR (data not shown). Toc64ΔTPR inhibits the import of wgt-pOE33 with a 5 times lower efficiency when compared to the full-length receptor (Fig. 10E, ΔTPR, lane 3-6; 10E, white triangle). Surprisingly, the translocation of pSSU was not affected by addition of Toc64TPR (Fig. 10E, black circle), but by addition of Toc64ΔTPR (Fig. 10E, white circle).

To confirm this result, the binding of the both preproteins to Toc64TPR and Toc64ΔTPR loaded matrices was investigated (5.2.7). In line with this, a strong association of wgt-pSSU with Toc64ΔTPR (Fig. 11A, lane 5; ΔTPR; Fig. 11C), but only a weak interaction with Toc64TPR was observed (Fig. 11A; lane 7, TPR; Fig11C). In contrast, wgt-pOE33 was recognized by Toc64TPR with high efficiency (Figure 2D; lane 7, TPR; Figure F lane 7, TPR), but less efficient by Toc64ΔTPR (Fig. 11A; lane 5, ΔTPR; Fig. 11C). The latter result is in line with the low capacity of Toc64ΔTPR to reduce the translocation of wgt-pOE33 into chloroplasts (Fig 10D; 10E). Analyzing the binding of reticulocyte lysate translated rtl-pSSU to Toc64 showed a similar association of pSSU with Toc64 (Fig. 11B, lane 3) as with Toc64ΔTPR (Fig. 11B, lane 5, Fig. 11C). In contrast, the interaction of rtl-pOE33 with Toc64TPR is reduced to the level of the recognition by Toc64ΔTPR when reticulocyte lysate translated preprotein is used in comparison to wgt-pOE33 (Fig. 11B, lane 5, compare with 11B, lane 3, 5, 7). In conclusion, the recognition of preproteins by the TPR domain of Toc64 is translation system dependent, whereas the recognition by the N-terminal domain is translation system independent (Fig. 11C).

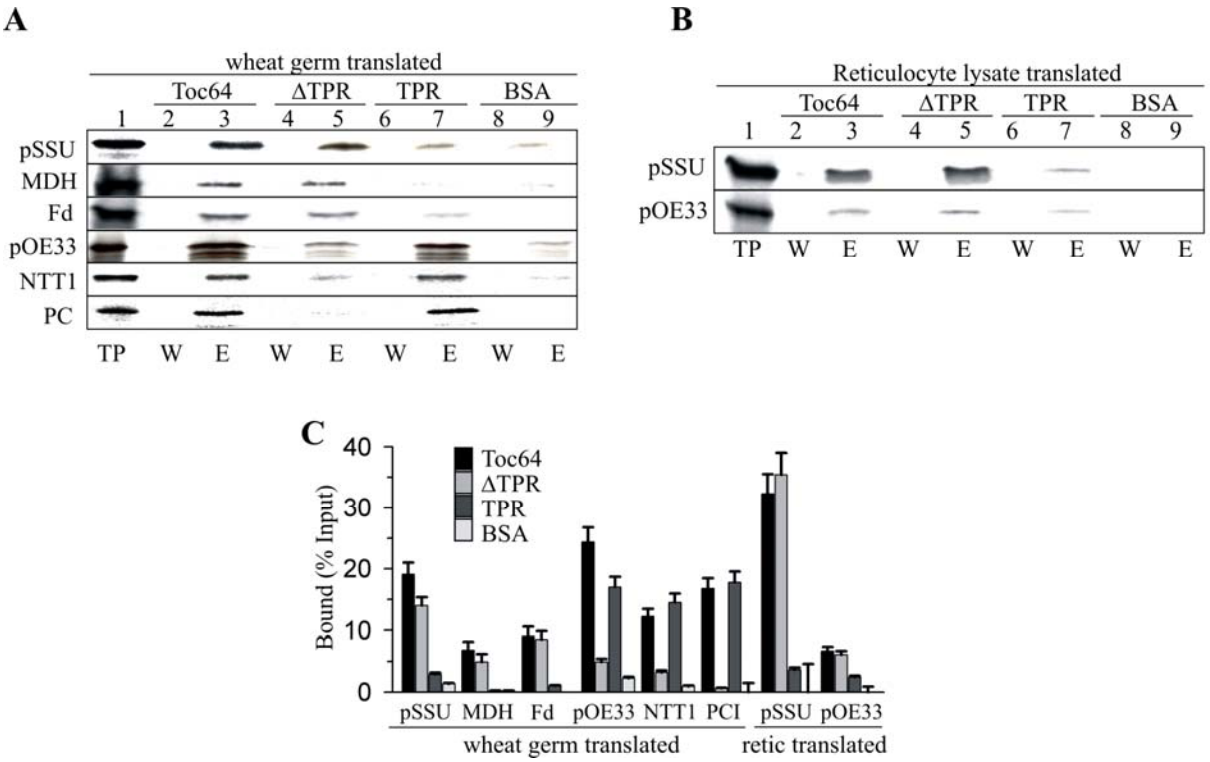

Figure 11. Preprotein recognition by Toc64.
A) Wgt S^{35}-labelled pSSU, MDH, Ferredoxin, pOE33, NTT1 or PC were incubated with a Toc64 (lane 2-3), Toc64ΔTPR (lane 4-5), Toc64TPR (lane 6-7) or BSA (lane 8-9) matrix. Proteins of wash (W) and elution fraction (E) are visualized. Lane 1 shows 20% of the translation product used (TP). **B)** Rlt-S^{35}-labelled pSSU or pOE33 (TP, lane 1, 100% loading) were incubated with a Toc64, Toc64ΔTPR (ΔTPR), Toc64TPR (TPR) or BSA matrix. Proteins of wash (W) and elution (E) are visualized. **C)** Quantification of multiple experiments is provided. Values are given in percent input.

To confirm that the observed differential recognition by Toc64 is not limited to pOE33 and pSSU, *in vitro* association of the nucleotide transport protein 1 (pNTT1), the precursor of the malate dehydrogenase (pMDH), ferredoxin (pFd) and plastocyanine 1 (pPC) with Toc64ΔTPR and Toc64TPR loaded matrices was analysed (Fig. 11A, 11C). Like for pOE33, a strong association of wgt-pNTT1 (inner envelope), pPC (Thylakoide) with the Toc64TPR affinity matrix was observed (Fig. 11A, NTT1, PC; Fig. 11C). In contrast, the stromal proteins pMDH and pFd behaved as pSSU; they bind with high efficiency to Toc64ΔTPR (Fig. 11A, MDH, Fd; Fig. 11C). The specificity of all the binding assays was confirmed by the absence of the preproteins association with the BSA coated column. Therefore, distinct preproteins contain additional information which results in recognition and targeting via Toc64, mainly the the cytosolic exposed TPR domain.

6.3.3 The interaction between Toc64TPR and preproteins is indirect

The observed differential affinity of the two domains of Toc64 was further explored for their ability to compete for the interaction between wgt-pOE33 or wgt-pSSU and full length Toc64. After binding of wgt-pSSU and wgt-pOE33 to Toc64 coated toyopearl matrix (5.2.7), the soluble expressed Toc64ΔTPR and Toc64TPR were added in order to compete for the interaction of Toc64 to the respective preprotein and subsequently elution of the preproteins from the affinity matrix. Here, Toc64TPR efficiently competed for the association between Toc64 and pOE33 (Fig. 12B, triangle), but not for the association between Toc64 and pSSU (Fig. 12B, circle). In line, significant higher concentrations of Toc64ΔTPR than of Toc64TPR are required for competition of the pOE33-Toc64 interaction (Fig. 12B, 12C, triangles). The opposite result was obtained for pSSU (Fig. 12A, 12B, circles), where Toc64ΔTPR compete efficiently pSSU-Toc64 interaction. Therefore, different domains of Toc64 facilitate the recognition of pSSU and pOE33.

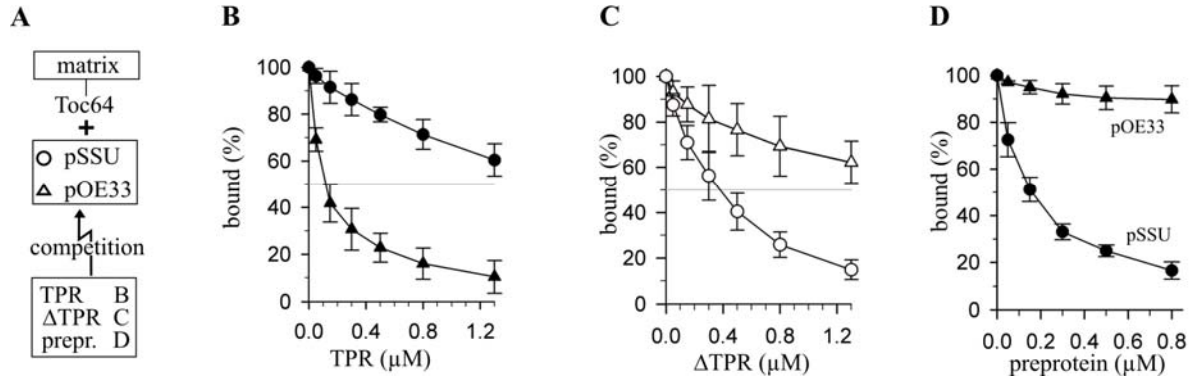

Figure 12. Preprotein recognition, competion and transfer.
A) The experimental scheme including the symbol legend is given**. B-D)** Wgt S^{35}-labelled pOE33 (triangle) or pSSU (circle) were incubated with a Toc64 matrix. After sufficient wash, bound proteins were eluted by increasing amounts of expressed Toc64TPR (B), Toc64ΔTPR (C), pSSU (for pSSU, D) or pOE33 (for pOE33, D). The amount of bound preprotein was quantified and is shown as percent of initial bound protein. Toc64TPR: black, Toc64ΔTPR: white.

To distinguish between a direct and an indirect interaction of the preproteins with Toc64, a matrix charged with Toc64 was incubated with wgt-pOE33 in the presence of heterologously expressed pOE33 as competitor (Fig. 12D). Here, only a weak competition was obtained (Fig. 12D, triangle) since about 90% of the wgt-pOE33 remained bound to Toc64 even in the presence of 0.8 μM pOE33. In contrast, expressed pSSU competed efficiently for the binding of wgt-pSSU to Toc64 ((Figure 12D, circle). This indicates that a specificity factor present in

the wheat germ lysate might mediate the interaction of wgt-pOE33 with the TPR region, wheras the association between wgt-pSSU seams to be direct to the N-terminal domain of Toc64. In conclusion, both domains within Toc64, the intermembrane space exposed region and the cytosolic exposed TPR, are involved in preportein recognition. The interaction between Toc64ΔTPR and the preproteins seems to be direct and translation system independent, whereas the interaction with Toc64TPR seems to be indirect and translation system dependent.

6.4 Function of the intermembrane space domain

6.4.1 The intermembrane space region of Toc64 recognises precursor proteins

The previous data demonstrated the existence of an intermembrane domain and also its involvement in complex assembly and that Toc64ΔTPR recognizes the rtl preproteines. The function of this intermembrane exposed region has been further addressed. At first, outer envelope membranes were treated with thermolysin (5.2.4) (Fig. 13A, lane 1), the 30 kDa protease resistant fragment was isolated (Fig. 13A, lane 2) and incubated with affinity matrices charged with different overexpressed preproteins pOE33 and pSSU (5.2.7) (Fig. 13A, lane 3, 4). In this experiment an interaction between both preproteins and the protease resistant 30 kDa domain was observed. In addition, the interaction of proteins targeted to the inner envelope with the intermembrane space exposed region of Toc64 was tested. The interaction of Toc64ΔTPR comprising the intermembrane space exposed domain with a phosphate carrier was investigated. For that, a dot blot with 13 amino acid long peptides, representing the entire sequence of the phosphate carrier, was incubated with expressed and purified Toc64ΔTPR. The interaction was analyzed by subsequent incubation with αToc64 antibodies (Reger, personal communication). The phosphate carrier was chosen since the protein contains a classical transit sequence and represents one of the smaller inner envelope proteins. Interestingly, Toc64 did not recognize the presequence of the phosphate carrier with highest efficiency but the loop region between the first and the second transmembrane region (Reger, personal communication). Subsequently the peptides with highest affinity for Toc64ΔTPR were synthesized. The sequences of the two regions with the highest binding affinity are given in Table 2. To test the specificity of these peptides, Toc64ΔTPR was incubated in the absence or presence of peptides with a pSSU coated matrix (5.2.7) since the interaction of the intermembrane space domain with this protein was previously demonstrated (Fig 13A). Here, the two peptides with the highest intensity in the dot blot assay efficiently

competed for an interaction between the receptor Toc64 and the precursor pSSU (Fig. 13B, lane 5, 7), whereas a control peptide did not compete for this interaction (Fig. 13B, lane 3).

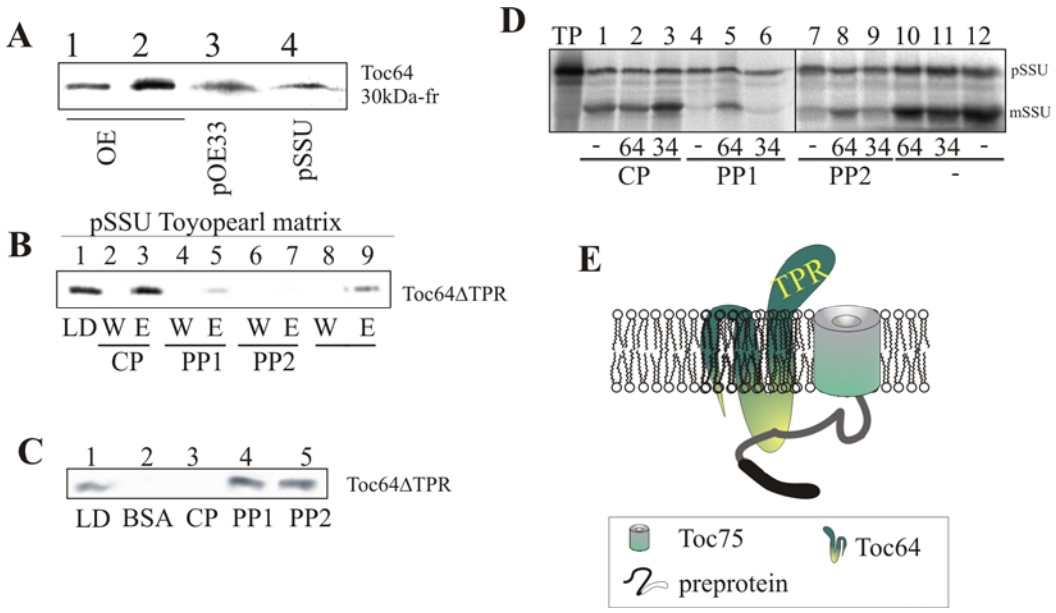

Figure 13. The properties of the intermembrane space domain of the Toc64 receptor.
(A) OEVs were incubated with thermolysin (lane 1), purified by sucrose density centrifugation, solubilized by addition of decylmaltoside (lane 2) and incubated with matrices coated with pOE33 (lane 3) or pSSU (Line 4). The eluted fraction (lane 3, 4) was collected, subjected to SDS-PAGE and immunodecorated by αToc64 antibodies. **(B)** Expressed and purified pSSU covalently coupled to a Toyo-Pearl matrix was incubated with Toc64ΔTPR (lane 1) in the absence (lane 8, 9) or presence of 20 µM of CP (lane 2, 3), PP1 (lane 4, 5) or PP2 (lane 6, 7). The wash (even numbers) and elution (odd numbers) are shown. **(C)** A thiol-sepharose matrix was incubated with BSA (lane 2), the CP (lane 3), PP1(lane 4) and PP2 (lane 5). The matrix was subsequently incubated with Toc64ΔTPR (lane 1, 10% load). The eluted proteins where subjected to SDS-PAGE, transferred to nitrocellulose membrane and immunodecorated by Toc64 antibodies. **(D)** Wgt S^{35}-labelled pSSU (TP: 10%translation product) was incubated for 10 minutes with chloroplasts (10 µg chlorophyll, lane 12) in the presence of 20 µM of the CP (lane 1-3), PP1 (lane 4-6) or PP2 (lane 7-9). Further, 10 µM of Toc64ΔTPR (lane 2, 5, 8, 10; 64) or Toc34ΔTM (lane (3, 6, 9, 11; 34) were added to the reaction. Imported protein was visualized by SDS-PAGE followed by autoradiography. The migration of the precursor form (pSSU) and the mature form (mSSU) is indicated. Control peptide (CP); peptide 1 (PP1); peptide 2 (PP2).
(E) A model of the action of the intermembrane space of Toc64 is presented.

To confirm the direct interaction between Toc64ΔTPR and the peptides, they were coupled to thiol-sepharose (5.2.7) and subsequently incubated with expressed receptor protein. As in the competition experiment, a specific interaction of Toc64ΔTPR with the two identified peptides was obtained (Fig. 13C, lane 4, 5) but not with a matrix coated with BSA or the control peptide (Fig. 13C, lane 2, 3). Next, the ability of the peptides to inhibit protein translocation of pSSU into chloroplast was analyzed (Fig. 13D). For that, pSSU was incubated with

chloroplasts in the presence of 20 µM peptides. The protein translocation was drastically reduced (Fig. 13D, Line 4, 7). However, when 10 µM Toc64ΔTPR and the peptides were added in parallel, the import was partially restored (Fig. 13D, lane 5, 8). This demonstrates that Toc64ΔTPR indeed recognizes the peptides, especially since Toc34 addition could not restore the protein translocation (Fig. 13D, lane 6, 9). The control peptide also inhibited the translocation of pSSU (Fig. 13D, line 1-3), but not to the same extent as the other peptides and the inhibition was not reduced in the presence of Toc64ΔTPR (Fig. 13D, lane 2). As previously shown in the section 6.3.1, Toc64ΔTPR and Toc34 competed for the translocation of pSSU when added (Fig. 13D). It can be concluded that Toc64ΔTPR, which contains the intermembrane space region, specifically recognizes the phosphate carrier at regions distinct from the transit peptide. Hence, the intermembrane space region of Toc64 is involved in preprotein recognition and translocation through the intermembrane space (Fig. 13E).

Peptide	Amino acid range	Amino acid composition
PP1	116-140	ylfnvifnilnkkiynyfpypyfvs
PP2	310-340	fadaiakvgmtkfisdlfwvgmfyhlynqla
CP	-	vapftglksaasfpc

Table 2. Peptides of phosphate carrier with affinity for Toc64 identified using dot blot analysis. Given is the amino acid range within phosphate carrier and the amino acid composition (the nomenclature as in figure 13). The sequence of the used synthetic peptides is underlined. Last line gives the sequence of the control peptide used.

6.5 Function of the cytosolic exposed TPR

After characterizing the involvement of the intermembrane space exposed region in preprotein recognition, the function of the cytosolic exposed TPR region was investigated. In the section 6.3.2, it was demonstrated that the recognition of preproteins by the TPR domain of Toc64 is translation system dependent and specific factor present in the wheat germ lysate might mediate this interaction. Furthermore, distinct preproteins contain additional information which results in recognition and targeting via the Toc64 TPR.

To characterise the factor present in wheat germ, which mediates the interaction between pOE33 and Toc64TPR, the effect of ATP addition to the Toc64-pOE33 interaction was analysed. Therefore, Wgt-pOE33 or wgt-pSSU were bound to Toc34 and Toc64 loaded affinity matrices (Fig. 14A), the ATP was used for elution and the fractions were analysed. Indeed, ATP eluted the bound wgt-pOE33 from Toc64 (Fig. 14A, lane 3), but not from Toc34. This suggests an interaction of Toc64 with ATP dependent factors, e.g. chaperones associated with pOE33 in the cytosol. It is known that the binding of ATP to the chaperones promotes

conformational rearrangements in the entire chaperone which results in changes in the affinity for peptide substrates (Buchberger et al., 1995, Panaretou et al., 1998). In contrast, wgt-pSSU bound to Toc64 was not eluted in the presence of ATP, supporting a differential mode of recognition by Toc64 (Fig. 14A).

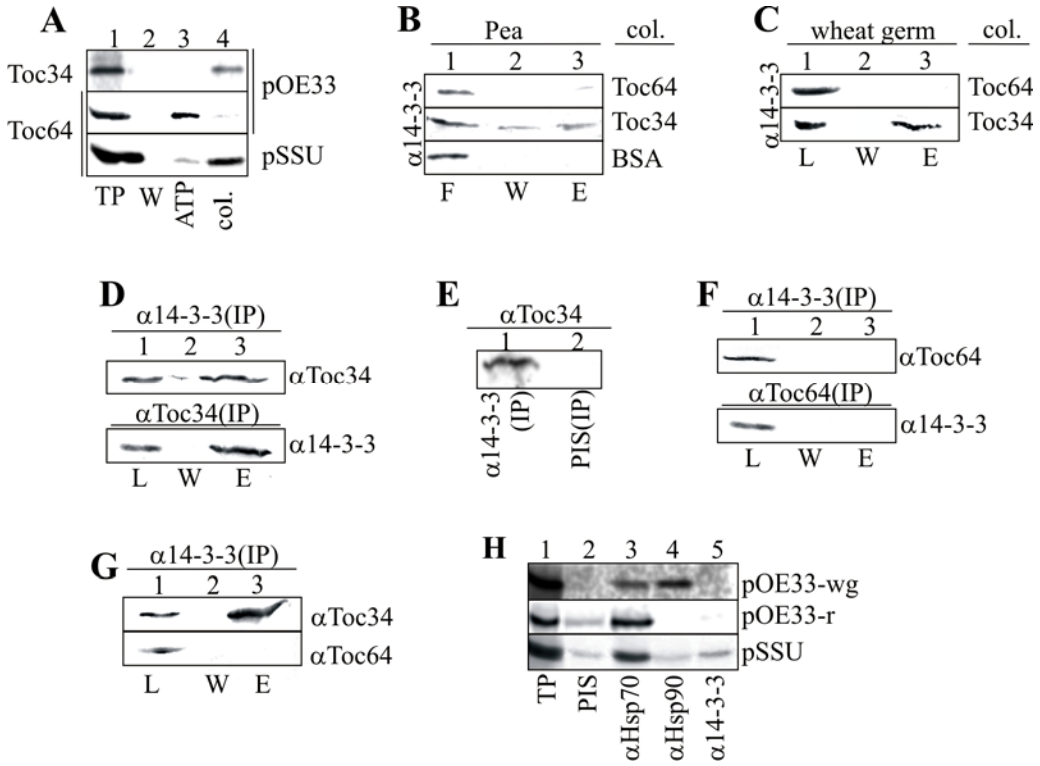

Figure 14. Toc64 recognises preproteins associated with chaperones.
A) Wgt S^{35}-labelled pOE33 or pSSU (lane 1, 20% of total) was bound to Toc34 or Toc64 affinity matrix and eluted by ATP. Proteins of wash (lane 2), elution (lane 3) and remaining on the column after ATP treatment (lane 4) were visualized. **B-C)** Cytosolic extract from pea (B) or wheat germ (C) was incubated with a Toc34, Toc64 or BSA matrix. Flow through (lane 1, B) or 10% loading (lane 1, C), wash (lane 2) and elution (lane 3) were immunodecorated using α14-3-3 antibodies. **D)** Expressed *at*14-3-3-ε was incubated with Toc34ΔTM (10% shown in lane 1), immunuprecipitated by α14-3-3 (top) or αToc34 (Bottom) antibodies. Proteins from the load, wash (lane 2) and elution (lane 3) were separated and immunodecorated with the indicated antibodies. **E)** As in D) but precipitated with α14-3-3 (lane 1) or preimmune serum (lane 2) and the precipitate decorated with αToc34 antibodies. **F)** Expressed *at*14-3-3-ε was incubated with Toc64 (10% shown in lane 1), precipitated by α14-3-3 (top) or αToc64 antibodies. Proteins from loaded, wash (lane 2) and elution (lane 3) were detected with the indicated antibodies. **G)** Expressed *at*14-3-3-ε was incubated with Toc64 and Toc34 together (10% shown in lane 1), precipitated by α14-3-3. Proteins from the load, wash (lane 2) and elution (lane 3) were separated by SDS_PAGE and immunodecorated with the indicated antibodies. **H)** Rlt-pOE33 (-r), wgt-pOE33 (-wg) or wgt-pSSU was precipitated by preimmune serum (lane 2), αHsp70 (lane 3), αHsp90 (lane 4) or α14-3-3 antibodies (lane 5). The precipitated proteins were visualized by autoradiography. (10% translation product, lane 1), IP: immunupecipitation.

6.5.1 Toc64 recognizes Hsp90-associated precursor proteins

The previous data indicate that the interaction of Toc64TPR with wgt-pOE33 is mediated by ATP dependent factors, like chaperones, interacting with the preprotein in the cytosol. Previously, it was speculated (Sohrt and Soll, 2000) that Toc64 might be the docking site for a cytosolic guidance complex for chloroplast preproteins, which consist of at least 14-3-3 and Hsp70 (May and Soll, 2000). To test this, soluble leaf extract from pea or wheat germ lysate was incubated with a Toc64, Toc34 or BSA loaded matrices (Fig. 14B, C) and the bound proteins were analysed. The major guidance complex component 14-3-3 interacts with Toc34 but not with Toc64 (Fig. 14B, lane 3; Fig.14C, lane 3). The interaction between 14-3-3 and Toc34 was further confirmed by incubation of soluble expressed Toc34 and 14-3-3 in solution and subsequent coimmunoprecipitation by α14-3-3 or αToc34 antibodies (Fig. 14D, lane 3). The interaction observed was found to be specific since the preimmune serum was not able to precipitate the complex (Fig. 14E). When Toc64 was preincubated with 14-3-3, no complex could be precipitated by α14-3-3 or by αToc64 antibodies (Fig. 14F, lane 3). To exclude an influence of Toc64 on the interaction of Toc34 with 14-3-3, both receptor proteins Toc64, Toc34 and 14-3-3 were mixed. Only Toc34, but not Toc64, was precipitated by the α14-3-3 antibodies (Fig. 14G, lane 3). In conclusion, Toc34 assembles the initial receptor for the guidance complex by direct recognition of 14-3-3.

So far, the data confirm that Toc64 interacts with wgt-pOE33 in an ATP-dependent manner (Fig. 14A) but does not recognize the guidance complex (Fig. 4B-G). To analyse which chaperones are associated with the preproteins in the translation system, wgt-pSSU, wgt-pOE33 or rlt-pOE33 (Fig. 14H) were immunoprecipitated using antibodies against 14-3-3 (Fig. 14H, lane 5), Hsp70 (Fig. 14H, lane 3) or Hsp90 (Fig. 14H, lane 4). All proteins were precipitated by Hsp70 antibodies, and only pSSU was precipitated by 14-3-3 antibodies. Here, wgt-pOE33, but not rtl-pOE33 was most efficiently precipitated by the antibodies against Hsp90 (lane 4) even though Hsp90 is present in reticulocyte lysate (Young et al. 2003) and which should be recognised by our commercial monoclonal antibodies. Hence, wgt-pOE33 seems to be associated with a complex distinct from the previously identified guidance complex (May and Soll, 2000). In general, preproteins are associated with Hsp70 in all translation systems, which is may be important to prevent aggregation of the preproteins.

To further test the chaperone/Toc64 interaction, a potent competitor of chaperone docking the C-terminal domain of *human*Hsp90 (aa566-732, C90, Young *et al,* 2003) was used. When wgt-pSSU (Figure 15A, black bar) or wgt-pOE33 (Fig. 15A, grey bar) were incubated with a Toc64 charged matrix in the presence of the C90 (Fig. 15A, Young *et al,* 2003), only the

interaction of pOE33 with Toc64 was reduced by 60%. In line with this observation, the import of pOE33 into chloroplasts (Fig. 15B, triangle), but not of pSSU was reduced in the presence of C90 (Fig. 15B, circle). This supports that pOE33 interacts with Toc64 via Hsp90 since C90 is known to compete for the recognition of Hsp90 by TPR domains (Young *et al*, 2003).

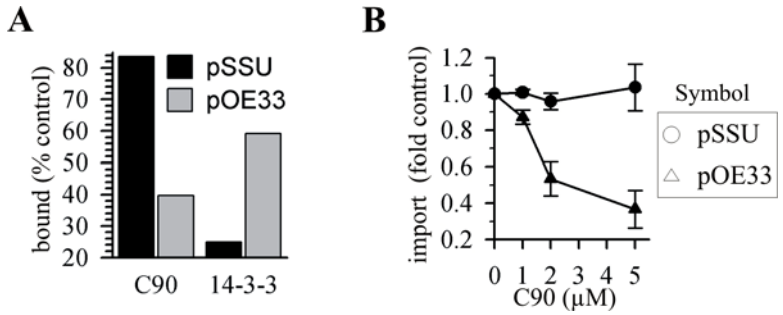

Figure 15. Toc64 recognises preproteins associated with Hsp90.
A) Wgt pSSU (black bar) or pOE33 (grey bar) was incubated with Toc64 matrix in the presence of the C-terminal domain of Hsp90 (C90) or 14-3-3-ε. The subsequent binding of preproteins was quantified and compared to the binding without the competitor. **B)** Translocation efficiency of pSSU (circle) or pOE33 (triangle) in the presence of increasing amounts of the C90 construct was quantified and compared to import without competitor.

Surprisingly, the association between pSSU and Toc64 loaded matrix was largely decreased in the presence of 14-3-3 (Fig. 15A, black bar), most likely due to a direct competition for binding sites within the transit sequence, since 14-3-3 does not interact with Toc64 (Figure 4B, C, F, G, lane 3), but with the preprotein (Fig. 14H, lane 5). 14-3-3 also reduced the association of pOE33 and Toc64 (Fig. 14H, grey bar) even though the effect of 14-3-3 was not pronounced as for pSSU. This might be explained by the similar fold of 14-3-3 and TPR domains (Das *et al*, 1998). 14-3-3 would therefore compete for Toc64TPR by recognition of the chaperones *in vitro* when added in chemical amounts. In conclusion, the association between Toc64 and wgt-pOE33 resembles the behaviour of a chaperone mediated interaction.

6.5.2 The Hsp90 is recognized by the TPR domain

The association of Hsp70 and Hsp90 to pOE33 (Fig. 14, 15) leads to the question which chaperone is recognised by Toc64. Therefore, soluble expressed Toc64, Toc64ΔTPR and Toc64TPR (5.1.3, Fig. 2A) were incubated with a matrix charged with a polypeptide reflecting the C-terminal portion of human Hsp70 (5.2.7) (C70, aa383-646, Figure 16A, lane 1-3) or Hsp90 (C90, Fig. 16A, lane 4-6). Toc64 and Toc64TPR bound to the C90 (Fig. 16, lane 6), but not to the C70 matrix (Fig. 16, lane 3). In addition, a molar excess of peptide

representing the 23 C-terminal amino acids of Hsp90 (P90, Fig. 16B, lane 4), but not the peptide representing the 25 C-terminal amino acids of Hsp70 (P70, Fig. 16B, lane 3) is able to compete for the interaction between Toc64TPR and the C-terminal construct of Hsp90 (C90). In contrast, Toc64 recognises both, Hsp70 and Hsp90 present in wheat germ lysate as determined by their interaction with a matrix charged with Toc64 or Toc64TPR proteins (Fig. 16C, upper panel). Here, Toc64 recognizes Hsp70 with higher efficiency than Hsp90 (Fig. 16C, lane 2), whereas the TPR region revealed a stronger interaction with Hsp90 (Fig. 16C, lane 4) suggesting that the transmembrane region present in Toc64 might act as a substrate for Hsp70. Silver staining of the fractions eluted from the Toc64 and Toc64TPR affinity matrices revealed the association of three proteins. The identity of the chaperones Hsp90 and Hsp70 were confirmed by immunodecoration and mass spectrometry (gi5123910, gi2827002). The protein of 50 kDa was identified as tubulin (Fig. 16C, square), however the specificity of this interaction remains elusive. Tubulin is the basic structural unit of microtubules, which are one of the components of the cytoskeleton.

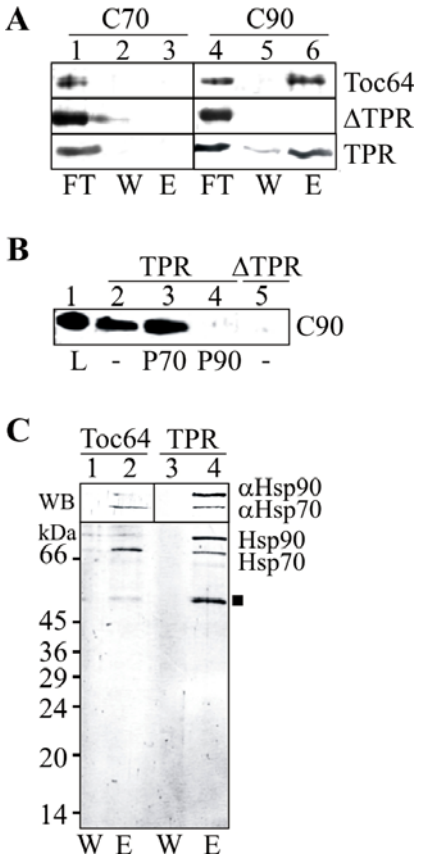

Figure 16. Toc64 specially recognizes Hsp90.
A) Toc64, Toc64ΔTPR (ΔTPR), or Toc64TPR (TPR) were incubated with a C70 (lane 1-3) or C90- matrix (lane 4-6). Flow through (FT), wash (W) and elution (E) were subjected to SDS-PAGE and visualized by silver staining. **B)** A Toc64TPR matrix was incubated with a synthetic peptide representing the C-terminus of Hsp70 (lane 3, P70) or Hsp90 (lane 4, P90) followed by incubation with 10 times lower molar amount of the C90 fragment (lane 2-4). As a control, Toc64ΔTPR charged matrix was incubated with C90 (lane 5). The loading (lane1) and elutions (lane 2-5) are shown. **C)** Wheat germ lysate was incubated with a Toc64 (lane 1, 2) or Toc64TPR (lane 3, 4; TPR) matrix. Proteins from wash (W) and elution (E) were either immunodecorated by αHsp70 or αHsp90 antibodies (WB, top) or silver stained (bottom). Square indicates tubulin.

To analyse the specificity of the chaperone interaction to Toc64TPR, wheat germ lysate was supplemented by additional cytosolic psHsp70 prior to incubation with Toc64TPR coated

matrix. Hsp70 was purified from a soluble extract of lysed plant cells and the purity of the chaperone was controlled by SDS-PAGE (data not shown). No competition of Hsp90 recognition by Toc64TPR was observed (Fig. 17A). In parallel, incubating increasing amounts of wheat germ lysate with a Toc64TPR matrix enhanced the interaction of Hsp90 with TPR, but not of Hsp70 (Fig. 17B). Further, incubating wheat germ lysate with the Toc64TPR matrix in the presence of the C90 construct reduced the interaction of Hsp90 to Toc64TPR but not of Hsp70 (Fig. 17C). All results together confirm that Hsp90 is recognised by Toc64 with higher affinity than Hsp70.

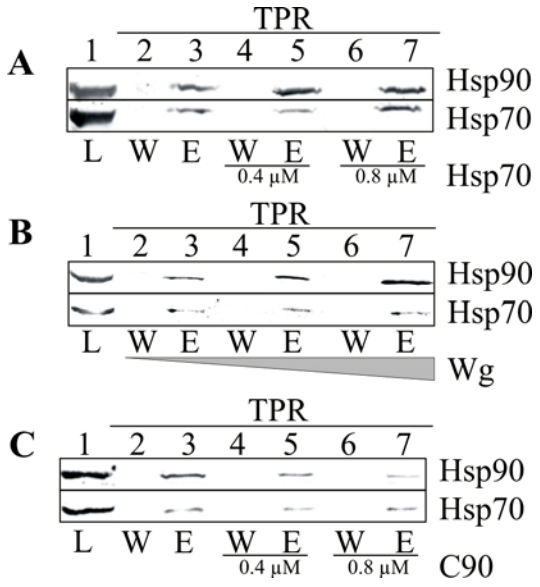

Figure 17. TPR of Toc64 recognizes specially Hsp90.
A-C) Wheat germ lysate (L, lane 1 shows 25%) was incubated with Toc64TPR matrix (lane 2-7, TPR) in the presence of 0.4µM (**A**, lane 4, 5) or 0.8 µM additional Hsp70 (**A**, lane 6, 7) or of 0.4 µM (**C**, lane 4, 5) or 0.8 µM of expressed C90 (**C**, lane 6, 7). In **B)**, the amount of wheat germ lysate used was 20 µl (B, lane 2, 3), 40 µl (B, lane 4, 5) or 100 µl (B, lane 6, 7; L, lane 1 shows 5µl wheat germ lysat). Proteins from final wash (W) and elution (E) were immunodecorated by αHsp70 (bottom) or αHsp90 (top).

Further, Toc64TPR matrix was incubated with either a soluble extract from pea, or with yeast extract (5.2.7). The association of pea Hsp90 to TPR was stronger (60% of total) in comparison to pea Hsp70 (15% of total) (Fig. 18A, line 3). Yeast Hsp90 interact with Toc64TPR, but not of with yeast Hsp70 (Fig. 18B, lane 3). In contrast, Toc64ΔTPR did not associate with yeast Hsp90 but to some extent with Hsp70 (Fig. 18B, lane 6). The latter finding might reflect a substrate recognition of Hsp70 since the construct (C70, aa383-646) not containing the ATPase domain does not associate with Toc64ΔTPR (Fig. 16A). Summarising, a basal recognition of Hsp70 was expected, especially since the C-terminus of plant Hsp90 and Hsp70 is closer related than these of fungal or mammal chaperones (Fig. 18C). The exposed clamp type TPR domain recognises a plant specific Hsp90 chaperone.

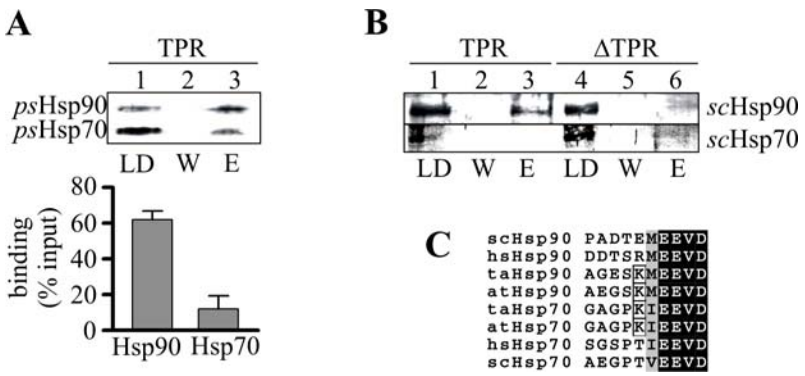

Figure 18. TPR of Toc64 recognizes a plant specific Hsp90.
A) Lysate from *P. sativum* (LD, lane 1 shows 25%) was incubated with a Toc64 matrix. Wash (lane 2) and elution (lane 3) were separated by SDS-PAGE and immunodecorated by αHsp70 or αHsp90 antibodies. The binding efficiency of Hsp90 or Hsp70 was quantified for at least 3 independent experiments and is given as percent of input. **B)** Yeast lysate was incubated with Toc64TPR (lane 2-4, TPR), or Toc64ΔTPR matrix (lane 5-7, ΔTPR). Loading (LD, lane 1,4 shows 25%), wash (W) and elution (E) were immunodecorated by αHsp70 or αHsp90. **C**) An alignment of the 10 C-terminal amino acids of representative Hsp70 or Hsp90 from human (Hs), yeast (Sc), wheat (Ta) or *A. thaliana* (At) is shown.

6.5.3 Toc64TPR behaves like a clamp type TPR

Tom70 (the outer membrane translocon in mitochondria) contains seven TPR motifs (Steger et al., 1990). Interestingly, it was shown that the three N-terminal TPR motifs have some similarity to a class of proteins that act as cofactors for the Hsp90 and Hsp70 chaperones in the folding process, involved in signal transduction (Young et al., 2001). Some of these cochaperones, typified as Hop, recognize the C-terminal sequences of Hsp90 and/or Hsp70 through specialized TPR domains (Scheufler et al., 2000). A dicarboxylate clamp in the TPR domain coordinates the C-terminal aspartate residue conserved in both chaperones, and the specificity for Hsp90 or Hsp70 is determined by hydrophobic contacts with neighbouring residues (Scheufler et al., 2000; Brinker et al., 2002).

The TPR domain of Toc64 assembles a clamp type fold as shown by its alignment with the TPR of Hop and Tom70 (Fig. 19A) and homology modelling (Mirus, personal communication). To test the prediction of the Toc64 TPR clamp type, point mutations specifically reducing the interaction of TPR with chaperones were introduced (Scheufler et al. 2000). Both $Toc64_{R550A}$ and $Toc64TPR_{N516A}$ were soluble expressed and used for in vitro binding assay (5.1.3, Fig. 2A; 5.2.7).

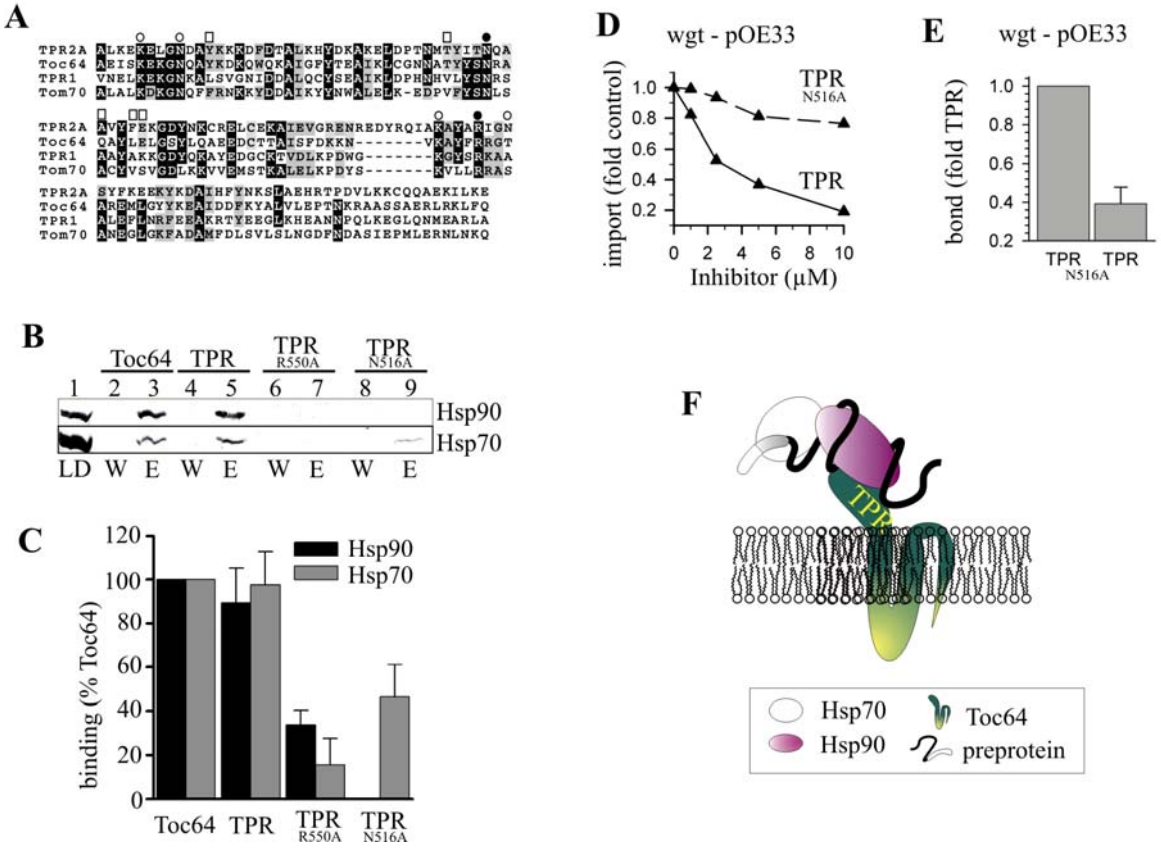

Figure 19. TPR of Toc64 is a clamp type and its interaction with chaperones is specific. **A)** An alignment of *ps*Toc64TPR (aa477-593), *hs*StyI (aa 4-120, TPR1, aa225-247, TPR2A) and *sc*Tom70 (aa99-213) is shown. Circles indicate positions of electrostatic interaction of TPR2A with the C-terminus of Hsp90 and squares indicate positions of hydrophobic interactions. Closed symbols mark amino acids mutated in Toc64. **B)** Wheat germ lysate (L, lane 1 shows 25%) was incubated with a Toc64 (lane 2, 3), TPR (lane 4, 5), TPR_{R550A} (lane 6, 7) or TPR_{N516A} (lane 8, 9) matrix. Proteins from final wash (W) and elution (E) were immunodecorated by αHsp70 or αHsp90 antibodies. **C)** At least 3 experiments as in C) were quantified and binding efficiency of Hsp90 (black) or Hsp70 (grey) normalised to interaction of the chaperones to Toc64**. D)** Chloroplasts (20 μg chlorophyll) were incubated with wgt S^{35}-labelled pOE33 in the presence of the indicated amounts of Toc64TPR (solid line) or $Toc64TPR_{N516A}$ (dashed line) at 25°C for 10 min. Translocation efficiency of pOE33 (triangle) was quantified and compared to import without competitor. The average of at least 3 independent experiments is shown. **E)** Wgt S^{35}-labelled pOE33 was incubated with Toc64TPR or $Toc64TPR_{N516A}$ matrix. The elutions were collected, the amount of the bound S^{35}-labelled pOE33 was quantified and normalized to association of pOE33 to Toc64TPR. **F)** The model of the interaction of Toc64TPR with the Hsp90 bound preproteins is shown.

In comparison to Toc64TPR, the interaction of $Toc64_{R550A}$ with Hsp90 or Hsp70 from wheat germ lysate was reduced by 70-80% (Fig. 19B, lane 3, 5, 7; Fig. 19C). In contrast, $Toc64TPR_{N516A}$ still recognises Hsp70, but not Hsp90 (Fig. 19B, lane 9, Fig. 19C). In line, $Toc64TPR_{N516A}$ was used as a competitor for the import of pOE33 into the chloroplast to discriminate the role of Hsp90 in wgt-pOE33. The competion of the import of pOE33 was

only very low (Figure 19D). Further, the efficiency of the recognition of wgt-pOE33 by Toc64TPR$_{N516A}$ loaded matrix was significantly reduced compared to wild type TPR (Fig. 19E). In summary, the clamp type TPR motif of Toc64 interacts with Hsp90 with high affinity but also with Hsp70, though with low efficiency. As determined previousely, pOE33 import into isolated chloroplasts was not affected by the presence of cBag (Fig. 10A). This C-terminal Bag domain of Bag-1 binds to the ATPase domain of Hsp70 to promote the exchange of ADP for ATP (Brehmer et al, 2001), which leads to the dissociation of the polypeptide from Hsp70. In contrast, cBag does not affect Hsp90/substrate interactions (Young and Hartl, 2000). Therefore, in case of an Hsp70 mediated interaction with Toc64, the presence of cBag should reduce the recognition and subsequent translocation of the preprotein. This is consistent with the observation that the import of rtl-pOE33 into chloroplasts was not reduced upon Toc64TPR addition (data not shown), since the preprotein was precipitated with Hsp70 antibodies but not with Hsp90 antibodies (Fig. 14H). Taken together, all these results suggest a receptor function of the TPR domain of Toc64 preferentially for Hsp90 bound preproteins (Fig. 19F).

6.5.4 The pOE33 guiding complex to Toc64

After demonstrating that pOE33 is associated with cytosolic chaperones (Fig. 14), which are recognised by Toc64 (Fig 16-19), the preprotein complex itself was analysed. It was reported that Hsp70 in its ADP-bound state exhibits a high affinity for substrate but the substrate association and dissociation rate is low (Mayer et al., 2000). The ATPase cycle of Hsp90 differs significantly from that of Hsp70. The Binding of ATP to Hsp90 causes only a slow release of substrate, and the conversion to the ADP state through hydrolysis of the bound ATP leads to fast release of substrate. Hsp90 in the ADP-bound state does not dissociate the complex with substrate (Young and Hartl, 2000).

Therefore, cell free wgt-pOE33 in the presence of ADP was subjected to size exclusion chromatography. The preprotein pOE33 eluted in a complex with an apparent molecular size of about 350 kDa (Fig. 20A, filled circles). Shifting the experimental conditions from 4°C to 25°C significantly decreased the amount of the complex purified (data not shown). With addition of ATP, which stimulates the dissociation of the chaperones, almost no complex was obtained (Fig. 20A, open circle). Comparing the distribution of pSSU translated in wheat germ (Fig. 20A, triangle) it becomes obvious that both proteins assemble different complexes.

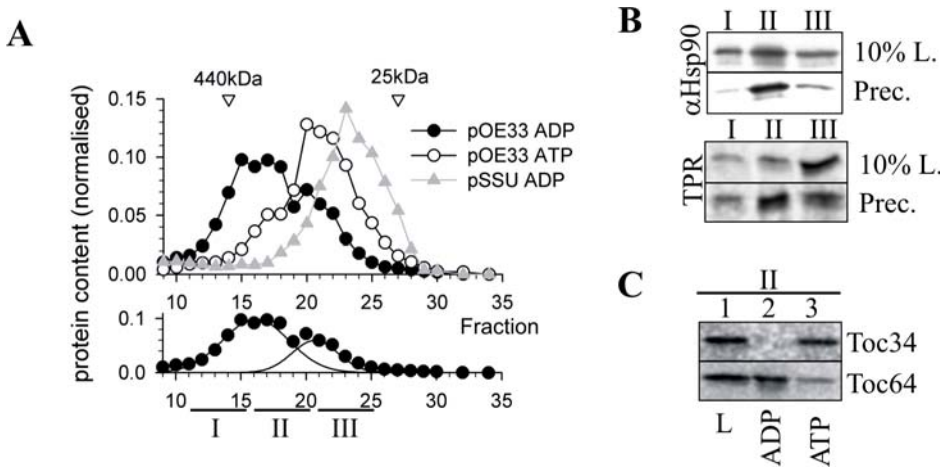

Figure 20. The pOE33 guiding complex.
A) Wgt S^{35}-labelled pOE33 (circle) or pSSU (grey triangle) was incubated with ADP (closed symbol) or ATP (open symbol) and applied onto Superdex 200 at 4°C. The amount of pOE33 in the indicated fractions was quantified. The column was calibrated with standard molecules for size exclusion chromatography (white triangle). On the bottom the distribution of the two peaks is indicated by a gaussian distribution (lines). **B)** Indicated fractions of the size exclusion separation (in A) were immunopreciptated by αHsp90 antibodies (top) or precipitated by incubation with Toc64TPR (bottom). Shown is 10% of the loading (10% L) and the precipitated fraction (Prec.). **C)** Fraction II of the size exclusion (see A, 10% shown in lane 1) was incubated with an affinity matrix charged with Toc34 (top) or Toc64 (bottom) after addition of ADP (lane 2) or ATP (lane 3). The latter lanes show the eluted protein.

The association of pOE33 with Hsp90 was subsequently probed by immunoprecipitating the protein from fractions of the gradient with antibodies against Hsp90 (Fig. 20B). The amount of protein precipitated was the highest in the peak fraction. However, a minor amount of protein was still precipitated from the fraction of higher and lower molecular weight. This might reflect the portion of complex assembled pOE33 in these fractions considering a normal distributions of complexes in a size exclusion experiment (indicated in Fig. 20A, bottom). In line pOE33 of the peak fractions binds to Toc64TPR, mainly pOE33 from the Fraction II binds efficiently (Fig. 20B, bottom). To probe if this complex can also be recognised by Toc34, the complex was incubated with ADP (Fig. 20C, lane 2) or ATP (Fig. 20C, lane 3) and with Toc34 or Toc64, respectively. As before, Toc64 was able to interact with the complex in the presence of ADP, but this binding was drastically reduced in the presence of ATP, which led to the disassembly of the complex (Fig. 20A). In contrast, Toc34 interacts with the preprotein only after the complex is dissociated (Fig. 20C, lane 2 vs. 3).

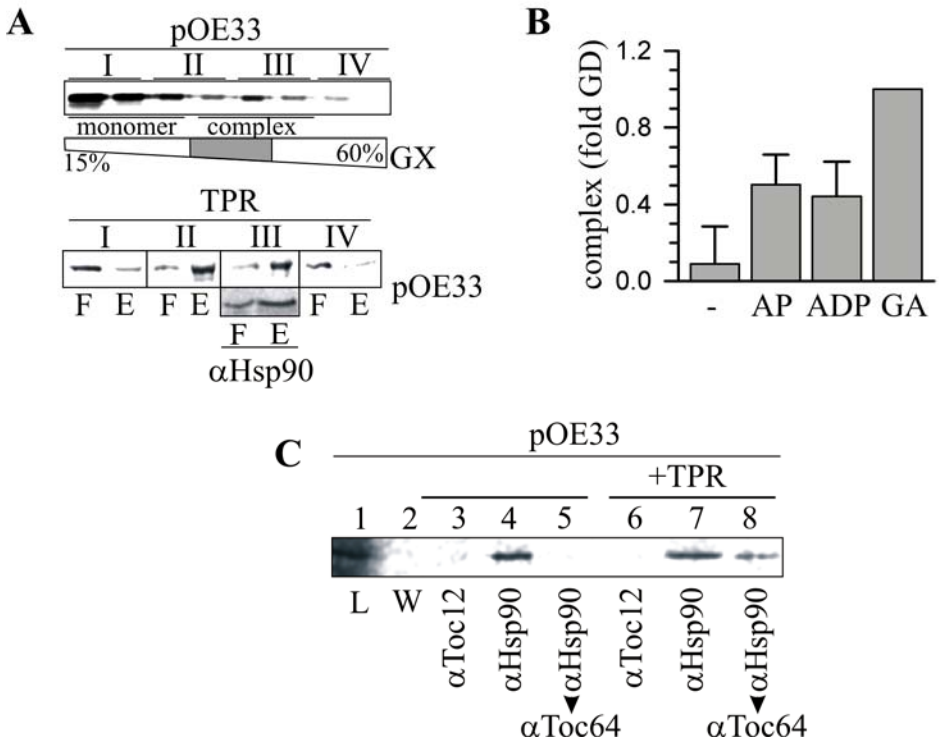

Figure 21. The pOE33 guiding complex interacts with TPR of Toc64.
A) Wgt S^{35}-labelled pOE33 was subjected to glycerol gradient centrifugation. The density direction is given at the bottom. The grey area indicates fractions where RubisCO was detected. Four fractions (I-IV) were incubated with a TPR-matrix (top panel, right). Flow through (F) and elution (E) are shown. pOE33 was subsequently immunoprecipitated from fraction III by Hsp90 antibodies (bottom). **B)** Wgt S^{35}-labelled pOE33 (-) was incubated with apyrase (AP), 1 mM ADP (ADP) or geldanamycin (GA) and subjected to glycerol gradient centrifugation. After fractionation, the amount of pOE33 in high molecular weight complexes was quantified and normalised to loading. The amount of complexed pOE33 is shown in relation to treatment with geldanamycin. **C)** Wgt S^{35}-labelled pOE33 (shown are 10%, L) was incubated with ADP in the absence (lane 3-5) or in the presence of Toc64TPR (lane 6-8, TPR) prior to immunoprecipitation by αToc12 (lane 3, 6) or αHsp90 antibodies (lane 4, 5, 7, 8). The precipitate of Hsp90 antibodies were further immunoprecipitated by antisera against Toc64 (αHsp90➔αToc64). Shown are one representative wash step (lane 2) and elutions.

To confirm the presence of the complex observed by size exclusion, wgt-pOE33 was subjected to glycerol gradient centrifugation in the presence of the Hsp90-specific inhibitor geldanamycin (GA), which blocks the ATP-driven chaperone cycle of Hsp90 with high affinity (Young and Hartl, 2000) (Fig. 21A). The pOE33 preprotein present in the peak fractions comigrating with the RubisCO complex (Fig. 21A, II, III, grey region) revealed a high affinity for Toc64TPR (Fig. 21A, right, top). In addition, an association of pOE33 present in fraction III of the glycerol gradient with Hsp90 could be demonstrated (Fig. 21A, III, bottom). However, this method is limited by the extended experimental time explaining

the lower abundance of the complex in comparison to the size exclusion purification. To test the stability of the complex, wgt-pOE33 was treated with apyrase, ADP or geldanamycin (GA), which is known to stabilize the substrate binding of Hsp90 specifically (Young and Hartl, 2000). After separation of wgt-pOE33 by a glycerol gradient, the largest amount of complex was found in the presence of geldanamycin (Fig. 21B), and the smallest without addition (Fig. 21B, -). In conclusion, complex formation is dependent on precursor recognition by Hsp90. So far, it was demonstrated that pOE33 is a part of the complex containing Hsp90, which is recognised by Toc64. To further demonstrate that the entire complex is indeed recognised by the TPR domain, wgt-pOE33 was co-precipitated by αHsp90 and αToc12 antibodies (the latter for control) in the absence or presence of Toc64TPR (Fig. 21C). As before, αHsp90 antibodies precipitated pOE33 efficiently (Fig. 21C, lane 4, 7) independent of the presence of the TPR. When the eluted proteins were precipitated by αToc64 antibodies, the formation of the complex could be confirmed (Fig. 21C, lane 8). Hence, Toc64 indeed recognises the cytosolic complex containing pOE33 and Hsp90.

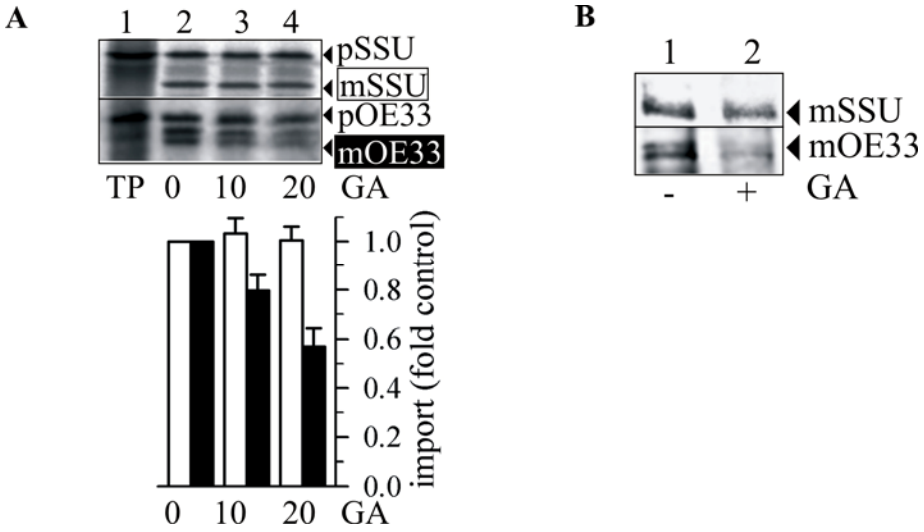

Figure 22. Preprotein translocation depends on recognition of Hsp90 by Toc64.
A) Wgt-pSSU (upper panel, white bar) or pOE33 (lower panel, black) were imported into isolated chloroplasts (20 µg chlorophyll) after mock (lane 2) or geldanaymycin treatment (lanes 3, 4) of translation product (lane 1 shows 10 %). Proteins of the re-isolated chloroplasts were separated by SDS-PAGE and visualised by phoshorimaging. The import efficiency (appearance of mature protein) quantified using AIDA software. The relation of import of geldanamycin treated to mock treated translation products is depicted. **B)** Tobacco protoplasts were transformed with plasmids encoding pSSU-GFP or pOE33-GFP. 16 hours after transfection radioactive labelled methionine was added and cells incubated for 2 hours in the absence (lane 1) or presence (lane 2) of geldanamycin. The mature protein was immunoprecipitated by GFP antibodies.

To demonstrate the participation of Hsp90 in targeting of preproteins to Toc64, wgt pSSU and pOE33 were imported into chloroplasts *in vitro* (Fig. 22A) either in the absence or presence of geldanamycin. In the presence of geldanamycin, a reduced translocation of pOE33, but not of pSSU, was obtained in both experiments. The translocation was reduced by 40% *in vitro* (Fig. 22A, quantification). The inhibition efficiency resembles the previously reported reduction of translocation of Tom70 dependent precursor into mitochondria in the presence of geldanamycin (Young et al. 2003). In addition, tobacco protoplasts were transformed by pOE33-GFP and pSSU-GFP constructs with subsequent pulse-chase labelling in the presence or absence of GA. After labelling, proteins were immunoprecipitated by antibodies against GFP. Like as observed for the *in vitro* import into chloroplast, the import of pOE33 *in vivo* was reduced in the presence of GA, but not of pSSU (Fig. 22B).

6.5.5 Depletion of the gene encoding for Toc64III impairs protein tanslocation efficiency

To further investigate the role of Toc64, a loss of function mutant for Toc64-III (At3g17970) was analysed (Fig. 23A). Arabidopsis plants of selected line 1 contained a homozygote T-DNA insertion since no gene specific PCR product could be obtained (Fig. 23B, lane 2). Furthermore, chloroplasts were isolated from the knockout plants (5.2.2) and subjected to SDS-PAGE and immunoblot analysis (Fig. 23C). These plants did not contain Toc64-III protein in the outer envelope (Fig. 23C), which can be explained by the absence of the transcript as analysed by RT-PCR on isolated mRNA from wild type or mutant plants (5.1.4) (Fig. 23D). The transcript level of *toc64-I* or *toc64-V* was not altered. However, these two proteins can not replace Toc64 in the outer envelope of chloroplasts since Toc64-V is a mitochondrial receptor and Toc64-I does not contain a TPR domain (Chew et al. 2004). In line with the previous report, the double-knockout mutant of *Physcomitrella patens* plants did not show a visible growth phenotype (Rosenbaum-Hofmann and Theg, 2005). *Physcomitrella patens* contains two proteins related to Toc64 (Hofmann and Theg, 2003). When the import of wgt-pOE33 into chloroplasts of the Arabidopsis wild type and the mutants lacking Toc64III protein was compared, the translocation of wgt-pOE33 associated with Hsp90 into chloroplasts from the knock out line was reduced about 40% compared to the import into the wild type (Fig. 23E and 23F, import and quantification). A similar reduction was reported for the translocation of proteins dependent on the TPR domain containing Tom70 in the deletion strain of this receptor (Young et al. 2003, Hinnes et al. 1990). In contrast, the translocation of rtl-pOE33, which was not associated with Hsp90 as confirmed in the immunoprecipitation

assay (Fig. 14H), into chloroplasts of *P. patens* Toc64 mutants was not altered in comparison to wild type (Rosenbaum-Hofmann and Theg, 2005).

Summarising, Toc64 is the receptor for preproteins delivered by a complex including the cytosolic Hsp90. This suggests a receptor function of the cytosolic exposed TPR domain of Toc64, preferentially for Hsp90 bound preproteins.

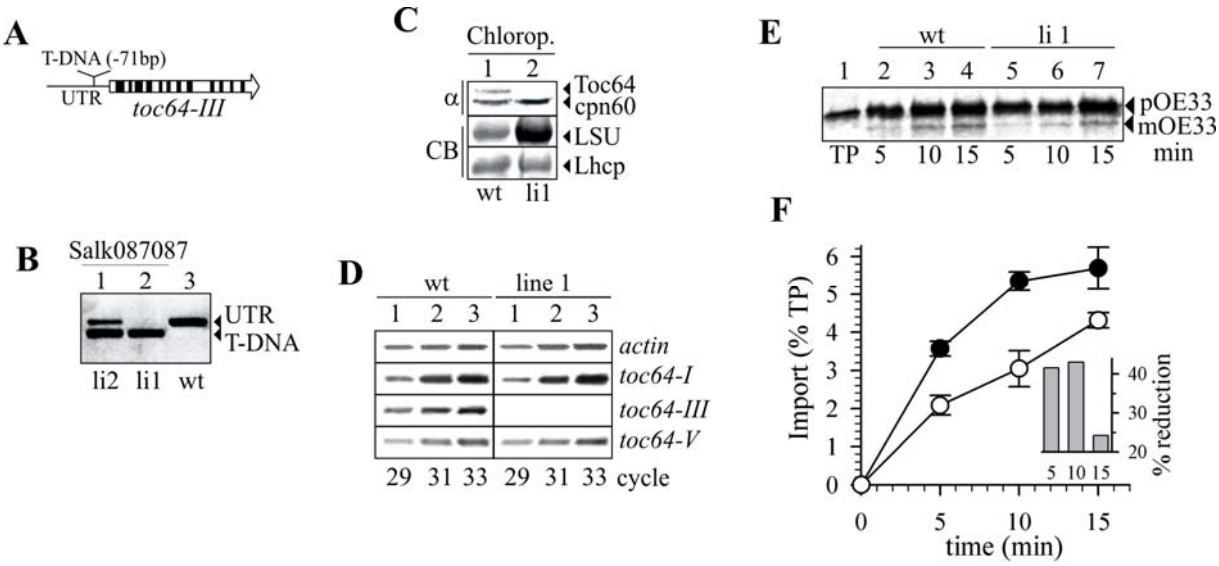

Figure 23. Depletion of Toc64 impairs preprotein translocation efficiency.
Plants of salk line 087087 (T-DNA insertion model in **A**) were grown on soil. **B)** T-DNA insertion was analysed by PCR using UTR specific and UTR/T-DNA specific primer pairs. **C)** Chloroplasts (10µg chlorophyll, of wild type (lane 1) or knock out plants (lane 2) were separated on SDS-PAGE, immunodecorated using indicated antibodies (top) or stained by coomassie blue (CB). **D)** Isolated mRNA was used for RT-PCR for the indicated number of cycles amplifying the indicated genes, actin is used as loading control). **E)** Wgt-pOE33 (lane 1, 10% translation product) was incubated with arabidpsis chloroplasts isolated from wild type (lane 2-4, wt), or knock out line 1 (lane 5-6, li 1) plants for the indicated times. **F)** The import efficiency (G) was quantified for 3 independent experiments. The reduction of pOE33 import into line 1 chloroplasts (open circles) compared to wild type chloroplasts (closed circles) is shown as inset.

6.5.6 Toc64 is not involvement in chloroplast movement

In the section 6.5.2 (Fig. 16C) it was observed that Toc64TPR interacts with tubulin from wheat germ lysate. The tubulin interaction might point toward a function of Toc64 in chloroplast movement. In general, it is believed that chloroplast movement is facilitated by actin filaments, not by tubulins (Wada, 2003).Therefore, the T-DNA insertion lines of Toc64III and Chup1 were analysed. Chup1 is a protein involved in chloroplast movement, as a T-DNA insertion leads to a loss of avoidance movement. This loss can be experimentally

analysed by illumination of a leaf section of wild type and knockout plants with a high light intensity (see Figure). In wild type plants the reorientation of chloroplasts can easily be seen, whereas in the knock out plants no difference to sections without illumination can be determined, which is a clear sign of the loss of avoidance movement. In contrast to the depletion of Chup1, the light exposure of leafs from the knockout Δ*toc64-III* plants leads to the typical avoidance movement as observed for wild type plants as assayed by the decolouration of the leaf in the exposed area. From that, it can be excluded that any gene targeted by a T-DNA insertion in the analysed knockout Δ*toc64-III* plant is involved in the chloroplast movement. Therefore, Toc64 is involved in protein transloction and the tubulin interaction is unspecific.

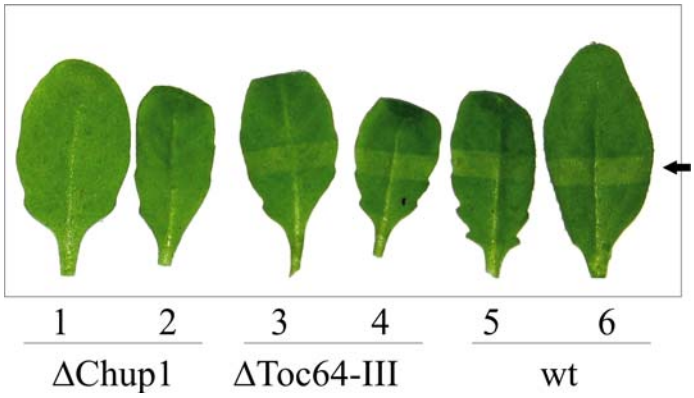

Figure 24. Toc64 is not involved in chloroplast movement.
The knockout Plants of Toc64III were grown on soil and leaves were harvested after 30 days. Immediately after harvesting, leaves were subjected to light exposure as described (Oikawa et al. Plant Cell 2003). For control, leafs from wt *A. thaliana* and from ΔChup 1 lines were exposed to the same light source. The results of the exposure are shown. Arrow shows the light exposed section of the leaves.

6.5.7 The functional association of Toc64 with Toc34

In the previous section it was shown that TPR domain of Toc64 is the docking site for Hsp90 delivered preproteins. Toc64 was linked to the Toc translocon by chemical cross-linking (Sohrt and Soll, 2000) and a dynamic association with the Toc core translocon was suggested (Becker et al., 2004a). It was demonstrated that Toc34GMP-PNP is the docking site for Toc64 in the Toc core complex and this interaction is mediated by the cytosolic exposed TPR domain of Toc64 (Becker, 2005). The question asked is, whether the preproteins bound to Toc64TPR will be transferred to Toc34 and how? It was demonstrated that Toc34 acts as a preprotein receptor (Jelic et al., 2002). To investigate preprotein transfer, wgt-pOE33 was

incubated with a matrix charged with Toc64 (Fig. 25A) or Toc34ΔTM in the presence of GMP-PNP (Fig. 25B). Subsequently, the binding of the precursor protein with the translocons was competed by soluble expressed Toc64 (black), Toc34 in the absence (white) or presence of GMP-PNP (grey). Soluble Toc64 protein efficiently competes for the interaction between Toc64 affinity matrix and pOE33 (Fig. 25A, black triangle). In contrast, the soluble Toc34 protein competed for the interaction between Toc64 affinity matrix and pOE33 only with low efficiency (Fig. 25A, white triangle) even in the presence of GMP-PNP (Fig. 25A, grey triangle).

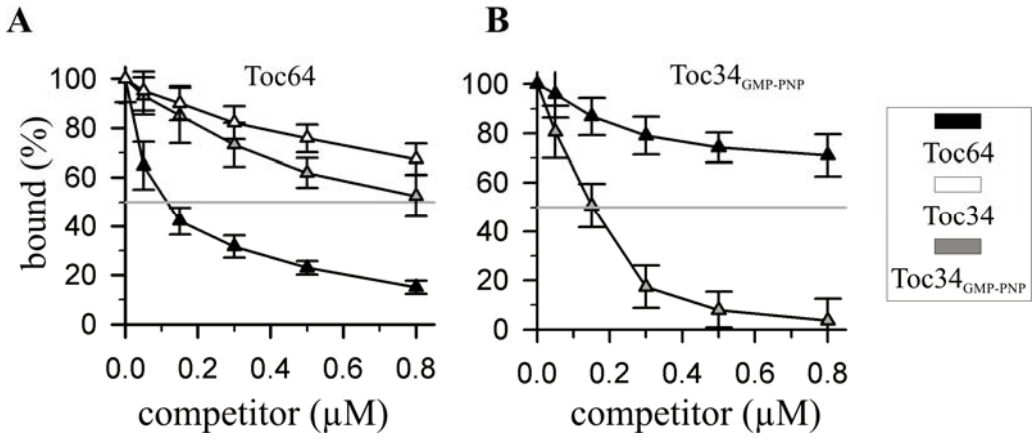

Figure 25.Preprotein transfer from Toc64 to Toc34.
Wgt S^{35}-labelled pOE33 was incubated with a Toc64 **(A)** or Toc34ΔTM coated matrix in the presence of 0.5 mM $MgCl_2$ and 1 mM GMP-PNP (**B)** followed by addition of increasing amounts of Toc64 (black) or Toc34ΔTM (white) loaded with GMP-PNP (grey). The amount of bound preprotein was quantified and is shown as percent of total bound protein. The average of at least 3 independent results is shown.

However, it was established previously that Toc34 in its GMP-PNP bound form recognises preproteins like pOE33 with high affinity. This interaction might be targeted toward the free preprotein, since the high affinity interaction was reconstituted *in vitro* as well (Schleiff *et al*, 2002). Hence, the competition experiment was performed with Toc34 as a bait. The competition of the complex between pOE33 and Toc34 by Toc34 in the absence or presence of GMP-PNP revealed a similar efficiency as found for the self-competition by Toc64 (not shown, Fig. 25B, grey triangle). Toc64 competed with a low efficiency for the interaction between $Toc34_{GMP-PNP}$ and pOE33 (Fig. 25B, black triangle). This finding supports that Toc34

recognises the precursor after dissociation from Hsp90, since Toc64 only recognises the chaperone associated precursor protein. The results suggest that an additional trigger is required for the release of pOE33 from Toc64 to Toc34. In a parallel study it could be demonstrated that a preprotein loaded Toc64 interacts with Toc34GMP-PNP and upon addition of ATP the preprotein is released from Toc64-TPR and afterwards can be recognised by Toc34GMP-PNP (Becker, 2005).

7. Discussion

7.1 Topology model of the Toc64

Recent work suggested that Toc64 contains an N-terminal transmembrane region, which is essential and sufficient for targeting to chloroplasts (Lee et al., 2004). Furthermore, it was documented that the N-terminal transmembrane region has an N_{in}-C_{out} orientation (Lee et al., 2004). In addition, the N-terminal hydrophobic domain of the two isoforms ppToc64-1 and -2 of *Physcomitrella patens* was necessary for interaction with chloroplasts, consistent with this region containing the transmembrane domain (Hofmann and Theg, 2005).

To date, the topology of the protein was based on prediction (Sohrt and Soll, 2000) and two experiments facilitating proteolysis of the proteins from *P. sativum* (Sohrt and Soll,2000) or *P. patens* (Hofmann and Theg, 2005) after import into the outer envelope of chloroplasts from *P. sativum* (Hofmann and Theg, 2005). However, topology prediction by programs has to be taken with care since the prediction quality of the programs should not be overestimated (Möller et al., 2001). For example, for the LHC proteins three transmembrane regions were obtained by crystallography (Kühlbrandt and Wang, 1991). Therefore, the database Aramemnon (Schwanke et al., 2003) was used to analyze this protein class in *A. thaliana*. For only one out of 13 proteins three transmembrane domains were assigned as consensus topology, whereas for three out of 13 only a single transmembrane region was proposed. Surprisingly, even for these three proteins single programs suggested three transmembrane regions. From this analysis it became clear that membrane spanning regions embedded in protein complexes or associated with ligands might well escape most of the prediction programs. Therefore, the three Toc64 proteins were analyzed regarding their predicted transmembrane regions by any available program (Fig. 5). In line with the observation for LHC proteins, the first transmembrane region, which was experimentally confirmed (Lee et al., 2004)**,** was not assigned to the *A. thaliana* protein (Fig. 5). However, the alignment of all three topological models considering all predicted transmembrane domains by individual programs indicate the presence of two further transmembrane spanning regions. Based on the obtained N_{in}-C_{out} orientation of the first transmembrane domain (Lee et al., 2004), in the new model the TPR domain is exposed to the cytosol, which is in line with previous results (Sohrt and Soll, 2000) and that the domain is in the supernatant after thermolysin digestion and subsequent carbonate extraction of the membrane (Figure 3-4). Furthermore, the topological model containing three transmembrane domains (Fig. 5) is very well supported by the obtained experimental results (Figure 3-7). First, the intermembrane space domain would be

about 30 kDa, the size of the protease resistant fragment observed after thermolysine treatment of right side out outer envelope vesicles and after import of Toc64 into intact chloroplast. This 30 kDa was not extractabe by carbonate treatment (Fig. 3, 7). Previously, a limited proteolysis of translation product (Hofmann and Theg, 2005) revealed a fragment stable against proteolytic digestion as well. Interestingly, this fragment was only observed for ppToc64-1 and not for ppToc64-2 even though a protease resistant fragment after translocation was obtained for both proteins, and the protease resistant fragment of the protein in the translation product migrates with a lightly different molecular weight (Hofmann and Theg, 2005). However, the stability of this protease resistant fragment in PpToc64 was not tested and essential controls to judge the membrane penetration of the protease are missing in this work. Second, the membrane protected fragment of Toc64 is a portion of the amidase and charged regions, which is confirmed by the staining of this stable fragment by antibodies recognizing these domains (Fig. 4). In contrast, antibodies against TPR do not recognize this stable fragment (Sohrt and Soll, 2000). Third, on the basis of the import of the radioactive labeled deletion constructs of Toc64 from *A. thaliana* the presence of the predicted transmembrane regions within the deleted domains are required for protein insertion. Since only Toc64 lacking the TPR domain or Toc64Δ1(1-437) revealed the same insertion and proteolytic behavior as observed for full length protein protein (Fig. 7). Fourth, the 30 kDa fragment was not accessible, since the size of the fragment did not change after the peggylation. Therefore, the cysteines within the stable fragment are protected by the membrane (Fig. 8). Based on the proposed topological model (Fig. 5), the peggylation results obtained for the full length protein can be explained by the modification of cysteine 110/507/534 of *P. sativum* and 117/531/558 of *A. thaliana* protein. The cysteines 110 and 117 are located at the N-terminal portion of Toc64 and the cysteines 507/534 and 531/558 are located in the cytosolic exposed TPR. The additional crosslink found for atToc64Δ1 can be explained by peggylation of either cysteine 144 or 424, which might be covered by the membrane and the cytosolic exposed domain in the other constructs. The topological results obtained are also in line with the results presented for PpToc64 from *P. patens* (Hofmann and Theg, 2005). For these proteins a resistant fragment was obtained after translocation. The presence of the protease resistant fragment in the translation product (Hofmann and Theg, 2005) could be well explained by protection by assisting chaperones or by folding of the domain, both not contradicting its location in the intermembrane space.

7.2 Toc64 is a component of the Toc translocon

Toc64 is a component of the Toc complex. It can be crosslinked to several subunits of the Toc complex (Sohrt and Soll, 2000). However, Toc64 was not copurified with the Toc core components in linear sucrose gradient. Hence, a dynamic association of the Toc core translocon was suggested (Sohrt and Soll, 2000, Becker et al., 2004a). It was demonstrated that Toc64 itself associates with the GTP-charged Toc complex by interaction of its TPR domain with the cytosolic exposed region of Toc34 (Becker, 2005) (Fig. 26). Hence, the modification of the isolation strategies can reveal the purification of different complexes, which was previously found for other translocation machineries like the Tom complex in the outer mitochondrial membrane (e.g. Künkele et al., 1998; Athing et al., 1999). The same was observed by BN-PAGE analysis, Toc64 assembles in a complex of about 700 kDa, which contains Toc159, Toc75, Toc34, Toc64, and Toc12. A further complex could be obtained at about 550 kDa containing the 86 kDa fragment of Toc159, Toc75, Toc34, Toc64 and Toc12 (Fig. 9). Therefore, two different forms of the Toc complex were obtained and both complexes contained Toc64 (Fig. 9). Interestingly, Toc64ΔTPR protein is assembled in chloroplasts in the same manner as Toc64 (Fig. 9). Therefore, the TPR domain is not required for the assembly of Toc64 into the complex. However, a size difference between the complexes containing Toc64 with or without TPR domain was not expected (Fig. 9).

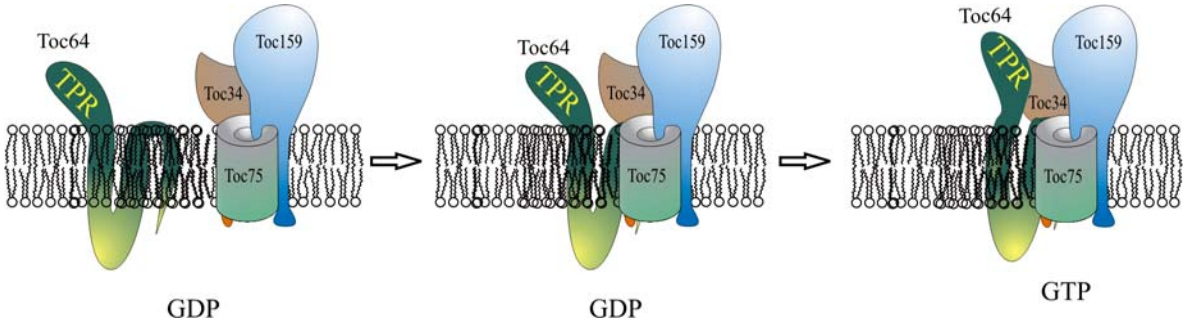

Figure 26. A model of GTP-dependent association of Toc64 to the Toc core complex is presented.

Interestingly, Tom70 of the mitochondrial outer membrane import machinery shares several features with Toc64. First, it dynamically associates with the Tom core complex (Künkele et al., 1998). However, this interaction has not been studied in detail. Second, it exposes a TPR-domain towards the cytosol. Third, the amount of Tom70 is two to five fold reduced in

comparison to the receptor components and Tom40 in the purified Tom translocon (Künkele et al., 1998). Moreover, as obtained for the Toc64-Toc34 interaction, an interaction between Tom20 and Tom70 was reported by chemical cross-linking and communoprecipiation *in situ* (Haucke et al., 1996).

7.3 Toc64 is a receptor for specific preproteins

The translocon at the outer envelope of chloroplasts contains at least two proteins discussed as receptors for chloroplastic precursor proteins, namely Toc34 and Toc64 (Soll and Schleiff, 2004). Toc34 acts as an initial receptor for preproteins either in monomeric form or delivered by the guidance complex (Figure 5, 9, 10; Becker et al. 2004). The receptor recognises both, the transit sequence (Schleiff et al. 2002) and the 14-3-3 of the guidance complex (Fig. 14) via a GTP regulated cytosolic domain (Schleiff et al. 2003). Furthermore the function of Toc64 was analysed. The receptor revealed differential recognition for wgt-preproteins (Fig. 10, 11). This differential recognition by Toc64 is not limited to wgt-pOE33 and wgt-pSSU, but to other preproteins as well. The wgt-pNTT1 (inner envelop localised protein), pPC (Thylakoid protein) bind strongly to Toc64TPR. In contrast, the stromal proteins pMDH and pFd behaved as pSSU (Figure 11). Therefore, distinct preproteins contain additional information which results in recognition and targeting via Toc64. One explanation might be the nature of the presequence, as all proteins not targeted via Hsp90 are located in the stroma. However, bared on 4000 proposed plastidic proteins the set of 6 proteins might be to limited to allow a statistical conclusion. In addition, Unlike for Hsp70, the exact structural features recognized by Hsp90 are not yet understood. In fulfilling its role, Hsp90 operates as part of multichaperone machinery in the cytosol, which includes Hsp70 and other cochaperones (Young et al., 2001 review). This interaction is mediated by the cytosolic exposed clamp type TPR domain (Fig 10, 11), which recognises Hsp90 chaperone (Fig. 14-17), but not the preprotein (Fig. 12). Therefore, ATP eluted the bound wgt-preprotein from Toc64 (Fig. 14), but not from Toc34. The Hsp90 interaction with Toc64 is in line with the observation that clamp type TPR domains recognise the C-terminus of the chaperones (Scheufler et al. 2000). Here, the domains recognising Hsp90 molecules reveal the highest affinity for C-terminal EEVD motif of Hsp90, which are highly conserved among all Hsp70 and Hsp90 molecules. Furthermore, domains recognising Hsp90 reveal a higher affinity for Hsp70 molecules than vice versa (Scheufler et al. 2000, Brinker et al. 2002). Therefore, in *in vitro* pull down experiments a basal recognition of Hsp70 by Toc64 was expected, whereas the TPR region revealed a stronger interaction with Hsp90 (Fig. 16). Additionally, the C-terminus of Hsp90

was able to compete for translocation (Fig. 15). Furthermore, Hsp90 interaction with Toc64TPR was not disrupted by addition of increasing amounts of Hsp70 (Fig. 17). Therefore, the clamp type TPR motif of Toc64 interacts with Hsp90 with high affinity but also with Hsp70 though with low efficiency (Fig. 15-18). However, an Hsp70 mediated interaction with Toc64 was excluded since the presence cBag during import into chloroplast does not reduce the recognition and subsequent translocation of the preprotein. It could be also demonstrated that Hsp90 indeed is involved in complex initiation between Toc64 and the precursor (Fig. 20-22) and this might be an alternative pathway.

Hsp70s from mammals or yeast are not recognised by Toc64TPR (Fig. 18). The different selectivity might be explained by the conserved lysine within the C-terminal portion of the plant Hsp70 and Hsp90 chaperones (Fig. 18C). Hsp70 of other species have the branched threonine at this position. Interestingly, yeast Tom70 interacts with Hsp70, whereas human Tom70 recognises both, Hsp70 and Hsp90, even though Hsp90 with higher affinity (Young et al. 2003). Further support for the influence of Hsp90 on import comes from the inhibition of the preprotein translocation into chloroplast by Geldanamycin (Fig. 22). The same result was obtained in the mammalian mitochondrial system. Therefore, the clamp type TPR domain of Toc64 builds an Hsp90 docking site receiving complexed preproteins.

Proteins containing TPR domain seem to be widely used in preprotein import in eukaryotic cells. Sec72 of the posttranslational import into the endoplasmatic reticulum and Pex5 of the peroxisomal protein translocation machinery are two receptor proteins with TPR domains (Gatto et al., 2000, Ponting 2000). However, an interaction with cytosolic chaperones of Pex5 and Sec72 remains to be investigated (Ponting 2000, Harper et al., 2003). Toc64 action parallels the action of Tom70. This chaperone preprotein targeting pathway may be compared with the posttranslational translocation of some soluble secretory proteins across the ER membrane (Rapoport et al., 1996). In the latter system, chaperones including Hsp70 keep the preproteins unfolded before translocation, but apparently no specific docking sites for chaperones are required for targeting. Instead, preproteins are recognized by components on the cytosolic face of the ER membrane, followed by translocation involving lumenal Hsp70 (Rapoport et al., 1996). In the Toc64 and Tom70 pathway, the functions of chaperone docking and preprotein recognition are combined within the receptors, perhaps reflecting a more active role of the cytosolic chaperones in preprotein import.

It was demonstrated that after the Hsp90 mediated preprotein recognition by TPR, the cytosolic exposed domain of Toc64, the preproteins are delivered to the core complex (Fig. 20; Becker, 2005). Toc64TPR itself is stably associated with the GTP-charged Toc complex by

interaction with the cytosolic exposed region of Toc34 (Fig. 25). The dissociation of the preprotein from the chaperone with ATP (Fig 14, 20) initiates its recognition by the second receptor of this pathway, Toc34 (Fig. 3, 14, 25; Becker, 2005). Such ATP dependent transfer of preproteins from Tom70 to Tom20 was also demonstrated *in vitro* (Komiya et al., 1997). Finally, delivery of the Hsp90 bounded preprotein from Toc64 to the core complex leads to the dissociation of Toc64TPR (Fig. 25), which is then open for a new uptake for Hsp90 guided preproteins. It still unclear how *in vivo* the chaperone dissociates from the receptor and by which mechanism are preproteins are transferred to Toc34. Hence, Toc34 acts as a general entrance receptor of the Toc core complex for incoming preproteins (Becker et al., 2004b). This observation is in line with the reduction of pOE33 import into chloroplasts from ΔToc33 knock out plants (Kubis et al. 2003). When compared to the translocon of mitochondria, the function of Toc64 parallels the action of Tom70 (Rehling *et al*, 2004). Both receptors are not essential as determined by knock out analysis (Young et al, 2003; Rosenbaum-Hofmann and Theg, 2005, Fig.23). Furthermore, a homologue of Toc64, but no homologue of Tom70, is found in the translocon of plant mitochondria (Chew *et al*, 2004). In turn, Toc34 takes over the function of two mitochondrial translocon subunits, namely Tom20, the initial receptor for mitochondrial preproteins, and Tom22, the initial docking site for all preproteins within the core complex (Rehling et al. 2004). The interaction of the preprotein with Toc34 stimulates its endogenous GTPase activity leading to a dissociation of Toc34 from the preprotein (Jelic et al., 2002; Becker et al., 2004b) and its transfer to Toc159. Here, the preprotein again induces GTP-hydrolysis of Toc159 by which the receptor pushes the preprotein through the translocation channel (Becker et al., 2004b).

In contrast to the initial steps of translocation across the outer membrane, almost nothing is known about the mechanism of translocation through the intermembrane space. The functional analysis of Toc64 implies that the intermembrane space domain is involved in the formation of an intermembrane space complex, which involves Toc64, Toc12 and an isHsp70 isoform (Becker et al., 2004a), and in preprotein recognition in the intermembrane space (Fig. 13). As for Toc64, a second preprotein recognition site different from the clamp-typeTPR domain in a 25 kDa core domain of Tom70 was reported (Brix et al., 2000). Analyzing the interaction of Toc64ΔTPR with the phosphate carrier localized in the inner envelope membrane revealed that in this case not the presequence but specific regions within the protein are recognized (Reger, personal communication). Threfore, it is thinkable that after crossing the Toc75 channel the preproteins are recognised by the intermembrane space region of Toc64 and possibly together with the intermembrane space components Toc12, isHsp70

and Tic22 which mediate either the transfer to the Tic translocons or insertion of the preprotein into the inner membrane. The existence of an intermembrane space complex in preprotein translocation in chloroplast seems to be reasonable, since the involvement of such system was already described for mitochondria. The 'small Tim' (translocase of the inner mitochondrial membrane) proteins have been found to participate by assisting precursor transport through the intermembrane space to the target complex either in the outer or inner membrane. Tim9/Tim10 and Tim8/Tim13 complexes keep the preproteins crossing the intermembrane space in a translocation competent state (Curran et al., 2002; Paschen et al., 2000).

8. Conclusion

Toc64 is a subunit of the chloroplast protein import machinery. In this work topological and functional properties of Toc64 were analysed. Moreover, a novel mechanism in which chaperones are recruited for a specific targeting event by a membrane-inserted receptor was outlined. Primary sequence analysis of Toc64 revealed three motifs. The first motif exhibits homology to prokaryotic and eukaryotic amidases, and is followed by the second motif, the charged region, and a C-terminal third motif the threefold repeated TPR-motif. The topological prediction of the protein by different programs revealed that Toc64 contains three transmembrane domains, which agrees well with the obtained results. All transmembrane regions are required for the correct topology of the protein. The protein has an N_{in}-C_{out} orientation (Lee et al., 2004). The TPR containing domain of Toc64 is cytosolic exposed, whereas a second domain of about 30 kDa is exposed to the intermembrane space and protected by the chloroplast outer envelope, which is a part of the amidase and charged regions.

Functional analysis demonstrated that Toc64 is a bi-functional preprotein receptor:

- The intermembrane space exposed domain participates in the formation of the intermembrane space complex, which involves Toc12, isHsp70 and Tic22 (Becker et al., 2004). This domain allows the association with the Toc complex and is involved in precursor protein recognition and translocation across the intermembrane space.
- The cytosolic exposed clamp-type TPR is the docking site for Hsp90 bound precursor proteins, with subsequent transfer to Toc34. This process resembles the preprotein recognition by Tom70 and delivery to Tom 20 in the mitochondrial import system.

In general, preproteins are associated with Hsp70 in all translation systems, which may be important to prevent aggregation of the preproteins. In the Toc64 pathway, the functions of chaperone docking and preprotein recognition are combined within the receptors, reflecting a more active role of the cytosolic chaperones in preprotein import.

9. References

Ahting U, Thun C, Hegerl R, Typke D, Nargang FE, Neupert W. and Nussberger S (1999) The TOM core complex: the general protein import pore of the outer membrane of mitochondria. *J. Cell Biol.* **147,** 959-968.

Aronsson H, Jarvis P (2002) A simple method for isolating import-competent Arabidopsis chloroplasts. *FEBS Lett* **529:** 215-220

Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, Schnell D, Kessler F (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis.*Nature* **403**: 203-207

Becker T, Hritz J, Vogel M, Caliebe A, Bukau B, Soll J and Schleiff E (2004a) Toc12, a novel subunit of the intermembrane space preprotein translocon of chloroplasts. *Mol. Biol. Cell.* **15**, 5130-5144.

Becker, T., Jelic, M., Vojta, A., Radunz, A., Soll, J. and Schleiff, E. (2004b) Preprotein recognition by the Toc complex. *EMBO J.* **23,** 520-530.

Becker T, Qbadou S, Jelic M and Schleiff E (2005) Let's talk about...chloroplast import. *Plant Biol.* **7**, 1-14.

Becker T (2005) Preprotein recognition and translocation by the Toc complex. *Dissertation*, LMU München: Fakultät für Biologie

Bedwell DM, Strobel SA, Yun K, Jongeward GD, Emr SD (1989) Sequence and structural requirements of a mitochondrial protein import signal defined by saturation cassette mutagenesis. *Mol Cell Biol*. **9:** 1014-25.

Brehmer D, Rüdiger S, Gassler CS, Klostermeier D, Packschies L, Reinstein J, Mayer MP, Bukau B (2001) Tuning of chaperone activity of Hsp70 proteins by modulation of nucleotide exchange. *Nature Struct Biol.* **8:** 427–432.

Brinker A, Scheufler C, Von Der Mulbe F, Fleckenstein B, Herrmann C, Jung G, Moarefi I, Hartl FU (2002) Ligand discrimination by TPR domains. Relevance and selectivity of EEVD-recognition in Hsp70 x Hop x Hsp90 complexes. *J Biol Chem* **277**: 19265-19275

Brix J, Zieglre GA, Dietmier K, Shneider-Mergener, Schulz GE, Pfanner N. (2000) The mitochondrial import receptor Tom70: identification of a 25 kDa core domain with a specific binding site for preproteins *J Mol Biol*. **303(4):** 479-88.

Buchberger A, Theyssen H, Schroder H, McCarty JS, Virgallita G, Milkereit P, Reinstein J, Bukau B. (1995) Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidence for interdomain communication. *J Biol Chem*. **270**: 16903-10.

Bukau B, Horwich A (1998) The Hsp70 and Hsp60 Chaperone Machines. *Cell* **92**: 351-366.
Bukau B, Deuerling E, Pfund C, Craig EA (2000) Getting newly synthesized proteins into shape. *Cell* **101:**119-122.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* **31:** 3497-3500.

Chew O, Lister R, Qbadou S, Heazlewood JL, Soll J, Schleiff E, Millar AH, Whelan J (2004) A plant outer mitochondrial membrane protein with high amino acid sequence identity to a chloroplast protein import receptor. *FEBS Lett* **557:** 109-114.

Cline K, Werner-Washburne M, Andrews J, Keegstra K (1984) Thermolysin is a suitable protease for probing the surface of intact pea chloroplasts. *Plant Physiol*. **75**: 675-678.

Curran SP, Leuenberger D, Schmidt E; Koether CM (2002) The role of the Tim8p-Tim13p complex in a conserved import pathway for mitochondrial polytopic inner membrane proteins. *J Cell Biol.* **158**: 1017-27.

Dabney-Smith C, van Den Wijngaard PW, Treece Y, Vredenberg WJ, Bruce BD (1999) The C terminus of a chloroplast precursor modulates its interaction with the translocation apparatus and PIRAC. *J Biol Chem* **274**: 32351-32359.

Deshaies RJ, Koch BD, Werner-Washburne M, Schekman R (1988) A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature* **33:** 800-5.

Das AK, Cohen PW, Barford D (1998) The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. *EMBO J* **17:** 1192-1199.

Demand J, Lüders J, and Jörg Höhfeld J (1998) The Carboxy-Terminal Domain of Hsc70 Provides Binding Sites for a Distinct Set of Chaperone Cofactors *Mol Cell Biol* **18**: 2023-2028.

Ferro M, Salvi D, Rievere-Rolland H, Vermat T, Seingneurin-Berry D, Grunwald D, Garin J, Joyard J, Rolland N (2002) Integral membrane proteins of the chloroplast envelope: identification and subcellular localization of new transporters. *Proc Natl Acad Sci USA* **99:** 1487-92.

Gatto, GJ, Jr, Geisbrecht, BV, Gould SJ. and Berg, JM (2000) Peroxisomal targeting signal-1 recognition by the TPR domains of human PEX5. *Nat. Struct. Biol* **7:** 1091-1095.

Georgoloulos C, Welch WJ (1993) Role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol*. **9:** 601-34.

Gutensohn M, Schulz B, Nicolay P and Flügge UI (2000) Functional analysis of the two *Arabidopsis* homologues of Toc34, a component of the chloroplast protein import apparatus. *Plant J* **23:** 771-783.

Hachiya N, Alam R, Sakasegawa Y, Sagaguchi M, Mihara K, Omura T (1993) A mitochondrial import factor purified from rat liver cytosol is an ATP-dependent conformational modulator for precursor proteins. *EMBO J* **12:**1579-86.

Hanahan D (1985) Techniques for transformation of E. coli. In.: DNA cloning I, a practical approach, Glover, D.M.; ed., *IRL PressLtd*, Oxford, 109-135.

Harper CC, Berg JM, Gould SJ (2003) PEX5 binds the PTS1 independently of Hsp70 and the peroxin PEX12. *J Biol Chem*. **278**:7897-901.

Haucke V, Horst M, Schatz G, Lithgow T (1996) The Mas20p and Mas70p subunits of the protein import receptor of yeast mitochondria interact via the tetratricopeptide repeat motif in Mas20p: evidence for a single hetero-oligomeric receptor. *EMBO J.* **15**:1231-7.

Hines V, Brandt A, Griffiths G, Horstmann H, Brutsch H, Schatz G. (1990) Protein import into yeast mitochondria is accelerated by the outer membrane protein MAS70. *EMBO J* **9** 3191-3200

Hinnah SC, Wagner R, Sveshnikova N, Harrer R and Soll J (2002) The chloroplast protein import channel Toc75: pore properties and interaction with transit peptides. *Biophys. J.* **83:**899-911.

Hofmann NR, Theg SM (2003) Physcomitrella patens as a model for the study of chloroplast protein transport: conserved machineries between vascular and non-vascular plants. *Plant Mol Biol.* **53:** 621-32.

Ivanova Y, Smith M.D, Chen K, and Schnell DJ. (2004) Members of the Toc159 import receptor family represent distinct pathways for protein targeting to plastids. *Mol. Biol. Cell* **15:** 3379-3392.

Jackson-Constan D and Keegstra K (2001) *Arabidopsis* genes encoding components of the chloroplastic protein import apparatus. *Plant Physiol* **125:** 1567-1576.

Jarvis P, Chen LJ., Li H, Peto CA, Fankhauser C, and Chory J (1998) An *Arabidopsis* mutant defective in the plastid general protein import apparatus *Science* **282:** 100-103.

Jarvis P, Robinson C. (2004) Mechanisms of protein import and routing in chloroplasts. *Curr Biol.* **14**: R1064-1077.

Jelic M, Sveshnikova N, Motzkus M, Hörth P, Soll J and Schleiff E (2002) The chloroplast import receptor Toc34 functions as preprotein-regulated GTPase. *Biol. Chem.* **383**, 1875-1883.

Jelic M, Soll J and Schleiff E (2003) Two Toc34 homologues with different properties. *Biochemistry* **42:** 5906-5916.

Joyard J, Block MA, Douce R (1991) Molecular aspects of plastid envelope biochemistry. *Eur J Biochem* **199**: 489-509.

Kessler F, Blobel G, Patel HA and Schnell DJ (1994) Identification of two GTP-binding proteins in the chloroplast protein import machinery. *Science.* **266**: 1035-1039.

Kessler F and Schnell DJ (2004) Chloroplast protein import: solve the GTPase riddle for entry. *Trends Cell Biol.* **14:** 334-338.

Kikuchi S, Hirohashi T and Nakai M (2006) Characterization of the Preprotein Translocon at the Outer Envelope Membrane of Chloroplasts by Blue Native PAGE. *Plant Cell Physiol.* **47(3)**: 363-71.

Komiya T, Rospert S, Schatz G and Mihara K (1997) Binding of mitochondrial precursor proteins to the cytoplasmic domains of the import receptors Tom70 and Tom20 is determined by cytoplasmic chaperones. *EMBO J.* 16: 4267-4275.

Komiya T, Mihara K (1996) Protein import into mammalian mitochondria. Characterization of the intermediates along the import pathway of the precursor into the matrix. *J Biol Chem*. **271(36)**: 22105-10.

Kouranov A, Chen X, Fuks B and Schnell DJ (1998) Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane. *J. Cell Biol.* **143**: 991-1002.

Kubis S, Baldwin A, Patel R, Razzaq A, Dupree P, Lilley K, Kurth J, Leister D, Jarvis P (2003) The Arabidopsis ppi1 mutant is specifically defective in the expression, chloroplast import, and accumulation of photosynthetic proteins. *Plant Cell*. **15:** 1859-1871

Kubis S, Patel R, Combe J, Bedard J, Kovacheva S, Lilley K, Biehl A, Leister D, Rios G, Koncz C, Jarvis P (2004) Functional specialization amongst the Arabidopsis Toc159 family of chloroplast protein import receptors. *Plant Cell.* **16(8)**:2059-77.

Kuhlbrandt W, Wang DN (1991) Three-dimensional structure of plant light-harvesting complex determined by electron crystallography. *Nature*. **350(6314)**:130-4.

Künkele KP, Heins S, Dembowski M, Nargang FE, Benz R, Thieffry M, Walz J, Lill R, Nussberger S and Neupert W (1998) The preprotein translocation channel of the outer membrane of mitochondria. *Cell.* **93:** 1009-1019.

Lee YJ, Sohn EJ, Lee KH, Lee DW and Hwang I (2004) The transmembrane domain of AtToc64 and its C-terminal lysine-rich flanking region are targeting signals to the chloroplast outer envelope membrane. *Mol. Cells.* **17**: 281-291.

Laemmli UK, (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**: 680-685.

Leister D. (2005) Origin, evolution and genetic effects of nuclear insertions of organelle DNA. Trends Genet. **21(12)**:655-63.

Lu J and Deutsch C (2001) Pegylation: a method for assessing topological accessibilities in Kv1.3. *Biochem.* **40:** 13288-113301.

Marshall JS, DeRocher AE, Keegstra K and Vierling E (1990) Identification of heat shock protein hsp70 homologues in chloroplasts. *Proc. Natl. Acad. Sci. USA.* **87:** 374-378.

May T, Soll J (2000) 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. *Plant Cell.* **12:** 53-64

Mihara K, Omura T (1996) Cytosolic factors in mitochondrial protein import. *Experientia*. **52(12)**: 1063-8.

Miras S, Salvi D, Ferro M, Grunwald D, Garin J, Joyard J, and Rolland N (2002) Noncanonical transit peptide for import into the chloroplast. J. *Biol. Chem*. **277:**47770-47778.

Moller S, Croning MD, Apweiler R (2001) Evaluation of methods for the prediction of membrane spanning regions. *Bioinformatics*. **17(7)**: 646-53.

Murakami H, Pain D, Blobel G (1988) 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J Cell Biol.* **107:** 2051-7.

Nada, A. and Soll, J. (2004) Evidence for a novel protein import pathway into chloroplasts. J. *Cell. Sci.* **117**: 3975-3982.

Nakrieko KA, Mould RM and Smith AG (2004) Fidelity of targeting to chloroplasts is not affected by removal of the phosphorylation site from the transit peptide. *Eur. J. Biochem*. **271:** 509-516.

Oblong JE and Lamppa GK (1992) Identification of two structurally related proteins involved in proteolytic processing of precursors targeted to the chloroplast. *EMBO J. **11(12):**4401-4409.*

Oikawa K, Kasahara M, Kiyosue T, Kagawa T, Suetsugu N, Takahashi F, Kanegae T, Niwa Y, Kadota A, Wada M. (2003) Chloroplast unusual positioning1 is essential for proper chloroplast positioning. *Plant Cell*. **15(12)**: 2805-15.

Palmer JD, Adams KL, Cho Y, Parkinson CL, Qiu YL, Song K (2000) Dynamic evolution of plant mitochondrial genomes: mobile genes and introns and highly variable mutation rates. *Proc Natl Acad Sci U S A*. **97(13)**: 6960-6.

Panaretou B, Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH (1998) ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *EMBO J*. **17(16)**: 4829-36.

Paschen SA, Rothbauer U, Kaldi K, Bauer MF, Neupert W and Brunner M (2000) The role of the TIM8–13 complex in the import of Tim23 into mitochondria. *EMBO J.* **19**: 6392-6400.

Pedrazzini E, Giovinazzo G, Bielli A, de Virgilio M, Frigerio L, Pesca M, Faoro F, Bollini R, Ceriotti A, Vitale A (1997) Protein quality control along the route to the plant vacuole. *Plant Cell*. **9(10):**1869-80.

Ponting CP (2000) Proteins of the endoplasmic-reticulum-associated degradation pathway: domain detection and function prediction. *Biochem. J*. **351:** 527-35.

Ramsey AJ, Russell LC, Whitt SR, Chinkers M. (2000) Overlapping sites of tetratricopeptide repeat protein binding and chaperone activity in heat shock protein 90. *J Biol Chem*. **275:**17857-62.

Rapoport TA, Jungnickel B and Kutay U (1996) Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu. Rev. Biochem.* **65**: 271–303.

Ratajczak T, Carrello A (1996) Cyclophilin 40 (CyP-40), mapping of its hsp90 binding domain and evidence that FKBP52 competes with CyP-40 for hsp90 binding. *J Biol Chem*. **271(6)**:2961-5.

Rial DV, Arakaki AK, and Ceccarelli EA (2000) Interaction of the targeting sequence of chloroplast precursor with Hsp70 molecular chaperones. *Eur. J. Biochem.* **276:**6239-6248.

Richter S, and Lamppa GK (1998) A chloroplast processing enzyme functions as the general stromal processing peptidase. *Proc. Nat. Acad. Sci. USA* **95:** 7463-7468.

Rehling P, Brandner K, Pfanner N (2004) Mitochondrial import and the twin-pore translocase. *Nat Rev Mol Cell Biol* **5**: 519-530

Rosenbaum-Hofmann N, Theg SM. (2005) Toc64 is not required for import of proteins into chloroplasts in the moss Physcomitrella patens. *Plant J* **43:** 675-687

Saiki RK, Gelfand DH, Stoffel B, Scharf SJ, Higuchi R, Horn GT, Mullis KB and Ehrlich HA (1998) Primer-directed enzymatic amplification of DNA with a thermo stable DNA polymerase. *Science* **239***:*487-491.

Schägger H, Cramer WA, and von Jagow G (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem*. **217**:222-230.

Scheufler C, Brinker A, Bourenkov G, Pegoraro S, Moroder L, Bartunik H, Hartl FU, Moarefi I (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* **101:** 199-210

Schleiff E and Klösgen RB (2001) Without a little help of "my" friends - Direct insertion of proteins into chloroplast membranes?. *Biochem. Biophys. Acta*. **1541**: 22-33.

Schleiff E, Soll J, Sveshnikova N, Tien R, Wright S, Dabney-Smith C, Subramanian C, Bruce BD (2002) Structural and guanosine triphosphate/diphosphate requirements for transit peptide recognition by the cytosolic domain of the chloroplast outer envelope receptor, Toc34. *Biochem.* **41**: 1934-1946.

Schleiff E, Soll J, Küchler M, Kühlbrandt W and Harrer R (2003a) Characterization of the translocon of the outer envelope of chloroplasts. *J. Cell Biol.* **160**: 541-551.

Schleiff E, Jelic M, Soll J (2003b) A GTP-driven motor moves proteins across the outer envelope of chloroplasts. *Proc Natl Acad Sci USA.* **100**: 4604-4609

Smith MD, Rounds CM, Wang F, Chen K, Afitlhile M and Schnell DJ (2004) atToc159 is a selective transit peptide receptor for the import of nucleus-encoded chloroplast proteins. *J. Cell Biol.* **165:** 323-334.

Sohrt K, Soll J (2000) Toc64, a new component of the protein translocon of chloroplasts. *J Cell Biol* **148**: 1213-12121

Soll J, Schleiff E (2004) Protein import into chloroplasts. *Nat Rev Mol Cell Biol* **5**: 198-208

Steger HF, Sollner T, Kiebler M, Dietmeier KA, Pfaller R, Trulzsch KS, Tropschug M, Neupert W, Pfanner N (1990) Import of ADP/ATP carrier into mitochondria: two receptors act in parallel. *J Cell Biol*. **111**:2353-63.

Sveshnikova N, Soll J and Schleiff E (2000) Toc34 is a preprotein receptor regulated by GTP and phosphorylation. *Proc. Natl. Acad. Sci. USA*. **97**: 4973-4978.

Schwacke R, Schneider A, van der Graaff E, Fischer K, Catoni E, Desimone M, Frommer WB, Flügge UI and Kunze R (2003) ARAMEMNON, a novel database for Arabidopsis integral membrane proteins. *Plant Physiol.* **131**: 16-26.

VanderVere PS, Bennett TM, Oblong JE, Lamppa GK (1995) A chloroplast processing enzyme involved in precursor maturation shares a zinc-binding motif with a recently recognized family of metalloendopeptidases. *Proc Natl Acad Sci U S A*. **92(16)**:7177-81.

Vojta A, Alavi M, Becker T, Hormann F, Kuchler M, Soll J, Thomson R, Schleiff E (2004) The protein translocon of the plastid envelopes. *J Biol Chem.* **279:** 21401-21405.

Waegemann K, Eichacker S and Soll J (1992) Outer envelope membranes from chloroplasts are isolated as right-side-out vesicles. *Planta.* **187**: 89-94.

Waegemann K, and Soll J, (1996) Phosphorylation of the transit sequence of chloroplast precursor proteins. *J. Biol. Chem.* **271**: 6545-6554.

Wada M, Kagawa T, Sato Y (2003) Chloroplast movement. *Annu Rev Plant Biol*. **54:**455-68.

Xiong J, Bauer CE (2002) A cytochrome b origin of photosynthetic reaction centers: an evolutionary link between respiration and photosynthesis. *J Mol Biol.* **322(5)**:1025-37.

Yang J, Roe SM, Cliff MJ, Williams MA, Ladbury JE, Cohen PT, Barford D (2005) Molecular basis for TPR domain-mediated regulation of protein phosphatase 5. *EMBO J* **24:** 1-10

Young JC, Obermann WM, Hartl FU (1998) Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90. *J Biol Chem*. **273(29):**18007-10

Young JC, Hartl FU (2000) Polypeptide release by Hsp90 involves ATP hydrolysis and is enhanced by the co-chaperone p23. *EMBO J.* **19:** 5930-5940

Young JC, Moarefi L, Hartl FU (2001 review) Hsp90: a specialized but essential protein-folding tool. *J Cell Biol* **154(2):** 267-73

Young BP, Craven RA, Reid PJ, Willer M and Stirling CJ (2001) Sec63p and Kar2p are required for the translocation of SRP-dependent precursors into the yeast endoplasmic reticulum *in vivo*. *EMBO J*. 20: 262-271

Young JC, Hoogenraad NJ, Hartl FU (2003) Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. *Cell* **112**: 41-50

Young JC, Agashe VR, Siegers K, Hartl FU (2004) Pathways of chaperone-mediated protein folding in the cytosol. *Nat Rev Mol Cell Biol* **5:** 781-791

Publications

Publications related to this work

***Qbadou S**, *Becker T, Mirus O, Tews I, Soll J, Schleiff E (2006) The molecular chaperone Hsp90 delivers precursor proteins to the chloroplast import receptor Toc64. *EMBO J.* **25(9)**:1836-47. *participate equally in this work.

Qbadou S, Becker T, Reger K, Soll J and Schleiff E. Toc64 recognises preproteins at both sites of the outer membrane of chloroplasts (submitted)

Others publications

Becker T, **Qbadou S**, Jelic M, Schleiff E. (2005) Let's talk about...chloroplast import. *Plant Biol (Stuttg)*. **7(1):**1-14. Review.

Chew O, Lister R, **Qbadou S**, Heazlewood JL, Soll J, Schleiff E, Millar AH, Whelan J. (2004) A plant outer mitochondrial membrane protein with high amino acid sequence identity to a chloroplast protein import receptor. *FEBS Lett.* **557(1-3)**:109-14.

Qbadou S, Tien R, Soll J, Schleiff E. (2003) Membrane insertion of the chloroplast outer envelope protein, Toc34: constrains for insertion and topology. *J Cell Sci*. **116**:837-46.

Acknowledgments

First of all, I would like to thank Prof. Dr. Soll for giving me an opportunity to perform this work and for helpful discussion and support.

I am especially grateful to my supervisor, PD Dr. Enrico Schleiff for helping me in many aspects of this work and being always generous with his time, and for his helpful advises as well as for his critical approach. Our numerous discussions helped me to develop a feeling for captivating facets of science.

I am very much thankful to Fatima, friend and colleague, for her support and patience during all the years.
I would like to thank Maike for her readiness to help, for the order and fun in the lab.
I would like to thank Alexander for careful reading of parts of this work.
I would like to thank Thomas for the nice cooperation and disscutions.
I would like to thank all members of the working group and Katharina, Mislav, Sunčana, Serena, Rolf and Oliver for the nice working atmosphere.
Specially, I would like to thank Katharina and Mislav for general informative discussions.
I would like to thank Eva and Lea for chloroplasts.
I would like to thank Rita Sharma for help in Protoplast preparation and for showing me the method of tobacco protoplast transformation.

My greatest thanks are dedicated to my parents and my family for supporting me, believing in me and much more.

Meinem Sohn danke ich für die Kraft, die ich durch ihn bekommen habe.
Meinem Mann danke ich für alles.

Curriculum vitae

Surname	Qbadou
Forname	Soumya
Data and place of birth	10.01.1971/Marokko
Marital status	Married, one child
1976 – 1988	Primary and secondary scool, Marrakech (Marokko)
1988 – 1993	Biology Study at the University Cadi Ayyad, Marrakech (Marokko). Final certificate: Licence
1994 – 1995	Germancours in Goethe-Institut, Rabat (Marokko). Certificat: Zertifikat Deutsch als Fremdsprache.
1995 – 1996	Germancours at the Christian Albrechts University, Kiel Certicat: Nachweis deutscher Sprachkentnisse.
1996	Start of the Biology study at the Christian Albrechts University of Kiel. (my university final certificate (Licence) from Morocco was only accepted as equivalent of Germany Vordiplom)
November 2000	Passing the diploma exam.
January 2001 – September 2001	Diploma thesis in cell biology in the working group of Prof. Dr. J. Soll at the Institut of Botany of the Christian-Albrechts-University of Kiel
February 2002	Start of the PhD- thesis in the working group of Prof. Dr. J. Soll at the Department of Botany at the Ludwig-Maximilians University of Munich

Ehrenwörtliche Versicherung

Die vorliegende Dissertation wurde von Dipl. Biol. Soumya Qbadou selbständig und ohne unerlaubte Hilfe angefertigt. Die Autorin hat zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder sich einer Doktorprüfung zu unterziehen. Die Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Ganzes vorgelegt.

Soumya Qbadou
München, den 30. 06. 2006