Protein Import into the Inner Envelope Membrane of Chloroplasts

Dissertation

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Abbreviations

Aa	Amino acid
ATP	Adenosine-5’-triphosphate
BSA	Bovine serum albumin
bp	Base pair
cap	m^7-Guanosin (5’) ppp (5’) Guanosin
C-terminus	Carboxy terminus
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GTP	Guanosine-5’-triphosphate
GTPase	Guanosine triphosphate hydrolase
HEPES	N-2-hydroxyethylpiperasin-N’-2-ethasulfonate
HP	Hypothetical protein
Hsp	Heat shock protein
IEP	Inner envelope membrane protein
IM	Inner envelope membrane
IPTG	Isopropylthiogalactoside
i	Intermediate of
MOPS	Morpholinopropansuphonate
m	Mature form of; mature protein
N-terminus	Amino terminus
NTP	Ribonucleoside-5’-triphosphate
$O.D._{600}$	Optical density at 600 nm
OM	outer envelope membrane
OE33	33-kDa oxygen evolving complex subunit
PCR	Polymerase chain reaction
PIC1	Permease in chloroplasts 1
Plsp1	Plastidic signal peptidase type I protease 1
PPT	Phosphoenolpyruvate translocator
p	Precursor of
RubisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SPP	Stromal processing peptidase
Tic	Translocon of the inner envelope membrane of chloroplast
Tim	Translocase of the inner mitochondrial membrane
Tim4	Translocation intermediate 4
Toc	Translocon of the outer envelope membrane of chloroplast
Tris	2-amino-2(hydroxymethyl)-1,3-propandiol
XPT	Xylulose-5-phosphate translocator

Summary

Most of the proteins localized in the chloroplast inner envelope membrane are synthesized on cytosolic ribosomes with a cleavable N-terminal chloroplast transit peptide. Most of them reach their final localization via the so called general import pathway consisting of the Toc complex at the chloroplast outer envelope membrane and the Tic complex at the chloroplast inner envelope membrane. Recent studies characterized precursor proteins which are targeted into the chloroplast inner envelope membrane by two different import pathways. The first route, called "conservative sorting", was described for Tic40 and Tic110, which prior to inner envelope membrane insertion reach the stroma. The second route, called "stop-transfer" was proposed for ARC6, which is arrested at the level of the inner envelope membrane and probably laterally inserted into the lipid bilayer. Taking into consideration both import mechanisms we characterized import pathways of nine chloroplast inner envelope membrane proteins containing cleavable transit peptides and a different number of hydrophobic α-helices. On the basis of the results observed in the stromal processing assays as well as results obtained in the pulse-chase experiments, within investigated precursor proteins two classes could be distinguished. The first class consisted of precursors processed once to their mature forms, *i.e*. containing a "single" transit peptide, whereas the second class consisted of precursors processed twice to the intermediate and the mature form, *i.e*. containing a bipartite transit peptide. In the processing of almost all precursor proteins stromal processing peptidase (SPP) was involved. Most probably at least one protein containing a bipartite transit peptide was also processed by another peptidase present not in the stromal compartment. We showed that despite of the differences in the number of hydrophobic transmembrane segments and different types of transit peptides, all investigated proteins had similar import properties. Their import was dependent on outer envelope membrane receptors and mediated by the general import pathway at least in the initial import phase. All investigated proteins required energy for import. 200 μM ATP was sufficient for proteins used in this study to achieve the maximal import rate. Interestingly, neither intermediates nor mature proteins were extractable from the membrane by urea treatment and all proteins seemed not to possess a soluble import intermediate. Therefore we claim that all investigated precursor proteins were imported via the "stop-transfer" pathway. Moreover, most probably at least some components of the Tic complex were involved in the transport of precursor proteins at the level of the inner envelope membrane and the process was Ca^{2+}/calmodulin regulated.

Zusammenfassung

Die meisten Proteine, die sich in der inneren Chloroplastenhüllmembran befinden, werden an den zytosolischen Ribosomen mit einem N-terminalen Transitpeptid gebildet. Die Proteine erreichen die innere Chloroplastenhüllmembran durch den so genannten Hauptimportweg, der aus dem Toc Komplex in der äußeren Chloroplastenhüllmembran und aus dem Tic Komplex in der inneren Chloroplastenhüllmembran besteht. In jüngster Vergangenheit wurden zwei Importwege in die innere Chloroplastenhüllmembran beschrieben. Es handelt sich hierbei um den so genannten „Conservativ sorting“, sowie den „Stop-transfer“ Importweg. Der „Conservativ sorting“ Importweg ist für Tic 40 und Tic 110 beschrieben worden. Die Proteine werden dabei zuerst in das Stroma importiert und dann in die innere Chloroplastenhüllmembran inseriert. Der „Stop-transfer“ Importweg ist für ARC6 vorgeschlagen worden. Gemäß dem Importmechanismus wird das Protein nicht in das Stroma importiert, sondern wird auf der Höhe der inneren Hüllmembran festgehalten und von dort direkt in die innere Chloroplastenhüllmembran eingebaut. Wir haben die Importwege von zehn Proteinen der inneren Chloroplastenhüllmembran charakterisiert. Sie besitzen Transitpeptide und eine unterschiedliche Anzahl an α-helikalen Transmembrandomänen. Aufgrund der Erkenntnisse, die wir durch stromale Prozessierungsuntersuchungen, sowie Pulse-Chase Experimente gewonnen haben, konnten wir die untersuchten Proteine gemäß ihrer Transitpeptidstruktur in zwei Klassen unterteilen: solche mit einteiligem oder zweiteiligem Transitpeptid. Die Proteine mit einem einteiligen Transitpeptid werden einmalig durch die stromale Prozessierungspeptidase (SPP) geschnitten. Die Vorstufenproteine mit zweiteiligem Transitpeptid werden zwei Mal, zunächst in ein Zwischenprodukt und daraufhin in ihre mature Form, prozessiert. Dies kann *in vitro* ebenfalls durch die SPP katalysiert werden, jedoch wird wahrscheinlich zumindest eines der Proteine mit einem zweiteiligen Transitpeptid durch eine weitere Protease, voraussichtlich aus einem anderen Chloroplastenkompartiment, prozessiert. Wir konnten zeigen, dass die untersuchten Vorstufenproteine trotz der unterschiedlichen Anzahl der hydrophoben Transmembrandomänen und der Transitpeptidstruktur ähnliche Importeigenschaften hatten. Ihr Import ist von den Rezeptoren der äußeren Chloroplastenhüllmembran abhängig und führt wenigstens in der anfänglichen Importphase durch den Hauptimportweg. Der Import der untersuchten Proteine benötigte Energie. 200 µM ATP war für die höchste Importeffizienz der untersuchten Vorstufenproteine ausreichend. Interessanterweise

konnten weder die Zwischenprodukte, noch die maturen Proteine durch 6 M Harnstoff aus der Membran extrahiert werden und alle Proteine scheinen nicht über ein lösliches Importintermediat zu verfügen. Darum kommen wir zu dem Schluß, dass die untersuchten Vorstufenproteine den „Stop-transfer“ Weg für ihren Import in die inneren Chloroplastenhüllmembran benutzen. Zudem ist mindestens ein Teil des Tic Komplexes am Import beteiligt und wahrscheinlich ist dieser Ca^{2+}/Calmodulin reguliert.

1. Introduction

1.1 Protein import into chloroplasts

Chloroplasts are the best characterized type of plastids in plant cells. It is widely accepted that they evolved from an ancient photosynthetic prokaryote similar to the present day cyanobacteria. This cyanobacterial ancestor was taken up by a heterotrophic host cell that already contained mitochondria (Cavalier-Smith, 2000). Due to the endosymbiotic event, chloroplasts are surrounded by two envelope membranes. Both the outer and inner chloroplast membranes are involved in the controlled exchange of a variety of ions and metabolites between the cytosol and the stroma (Joyard *et al*., 1998). The outer envelope membrane of chloroplasts contains a number of regulated and specific pore proteins that originated most likely from the outer membrane channel proteins of the cyanobacterial ancestor. They act as selectivity filters that allow passage of small molecules (Bölter and Soll, 2001). At the inner envelope membrane different translocators mediate the exchange of metabolites. These translocators coordinate cytosolic and stromal metabolic processes like photosynthesis, photorespiration, biosyntheses of sucrose, starch and amino acids (Flügge and Heldt, 1991). Apart from pores and metabolite translocators at the outer and inner envelope membrane, respectively, there are two proteinaceous complexes that mediate translocation of proteins synthesized in the cytosol into chloroplasts. The Toc complex (translocon at the outer envelope of chloroplasts) consists of five proteins: Toc159, Toc75, Toc64, Toc34 and Toc12, and mediates the initial recognition of preproteins and their translocation across the outer envelope membrane (Schnell *et al*., 1997; Schleiff *et al*., 2003a; Becker *et al*., 2004a). The Tic complex (translocon at the inner envelope of chloroplasts) is made up of seven proteins: Tic110, Tic62, Tic55, Tic40, Tic32, Tic22 and Tic20 (Benz *et al*., 2007). Tic110 physically associates with the Toc complex (Akita *et al*., 1997) and provides a membrane translocation channel for the inner membrane (Heins *et al*., 2002). The Toc and Tic complexes import precursor proteins into the chloroplasts via the so-called general import pathway (Cline and Henry, 1996; Soll and Schleiff, 2004; Gutensohn *et al.*, 2006) through which most of the inner membrane, stromal and thylakoidal proteins are imported. However, alternative import pathways are suggested since the import routes which do not involve the proteins of the general import machinery were described for some proteins: Tic32 (Nada and Soll, 2004) and chloroplast envelope quinone oxidoreductase homologue, ceQORH (Miras *et al.*, 2002; 2007), which

do not contain a cleavable presequence and are targeted to the inner envelope membrane without involvement of components of the general import pathway as well as for stromal protein α-carbonic anhydrase, CAH1, that involves secretory pathway for its import into chloroplasts (Villareyo *et al*., 2005; Faye and Daniell, 2006).

In general, the translocation of preproteins across the chloroplast envelope is an energy-dependent process (Theg and Scott, 1993). For the binding of precursor protein to the organelle low energy concentration (<50 μM NTP) is required. Hydrolysis of higher energy concentration (>100 μM NTP) translocates the precursor protein across the chloroplast envelope membranes (Vothknecht and Soll, 2000; Soll and Schleiff, 2004).

The import process of nuclear-encoded preproteins into chloroplasts is highly regulated. In the cytosol and at the outer envelope membrane of chloroplasts regulation involves phosphorylation/dephosphorylation of transit peptides (Waegemann and Soll, 1996), GTP/GDP cycles of the Toc34 and Toc159 receptors (Schleiff *et al*., 2003b) as well as the action of molecular chaperones Hsp70, Hsp90 and 14-3-3 proteins (May and Soll, 2000; Qbadou *et al*., 2006). At the inner envelope membrane and in the stroma import is probably regulated by Ca^{2+}/calmodulin (Chigri *et al*., 2005) as well as by the redox state of the organelle. Three proteins of the Tic complex could be involved in this redox regulation, namely Tic62 (Küchler *et al*., 2002), Tic55 (Caliebe *et al*., 1997) and Tic32 (Hörmann *et al*., 2004).

As precursor proteins emerge from the translocation channel, the transit peptide is cleaved off by a stromal processing peptidase (SPP) (Richter and Lamppa, 1999). The protein reaches its functional conformation with assistance of molecular chaperones or is further directed to other sub-compartments of the chloroplasts by additional targeting signals (Jarvis and Robinson, 2004).

1.2 Transit peptides

Most proteins directed into chloroplasts are synthesized on cytosolic ribosomes with an N-terminal extension called presequence or transit peptide. Transit peptides of chloroplast proteins prevent mistargeting into other cell compartments and are mostly required for sorting to the correct intraorganellar location (Bruce, 2000; 2001; Soll and Schleiff, 2004). They vary considerably in length (from about 30 to 120 residues) and amino acid sequence. Transit peptides of chloroplast proteins have properties that

superficially resemble presequences of mitochondrial precursor proteins, *i.e*. they are rich in hydroxylated residues and deficient in acidic residues. Chloroplastic transit peptides are divided into three domains: an amino terminal 10–15 residues devoid of Gly, Pro, and charged residues, a variable middle region rich in Ser, Thr, Lys, and Arg and a carboxy-proximal region with a loosely conserved sequence (Ile/Val-x-Ala/Cys_Ala) for proteolytic processing (Cline and Henry, 1996).

Import into the inner envelope membrane of the vast majority of known inner envelope proteins requires a cleavable transit peptide. There are two possible ways of insertion already known for mitochondria (Hartl and Neupert, 1990), which are proposed as well for chloroplasts on the basis of similarities of their import machineries. According to the "conservative sorting" import pathway preproteins are directed into the stroma, processed by SPP and re-exported into the inner envelope membrane. This way of insertion is represented by Tic110 (Lübeck *et al*., 1997; Vojta *et al*., 2007) and Tic40 (Tripp *et al*., 2007). The cleavable presequence of Tic110 directs the preprotein into the stroma whereas inner envelope targeting information is contained within a hydrophobic segment of the mature protein sequence (Lübeck *et al*., 1997). Tic40 contains a bipartite cleavable transit peptide at its N-terminus and similarly to Tic110, this part of the protein is responsible solely for stroma targeting. Insertion of Tic40 into the inner envelope membrane is determined by a serine/proline-rich domain positioned before the only transmembrane domain (Tripp *et al*., 2007). The second way of insertion into the inner envelope membrane, the "stop transfer" pathway, is used by preproteins containing sequence motifs that induces exit of the preprotein from the import machinery at the level of the inner envelope membrane. The "stop transfer" mechanism in chloroplasts was proposed by Flügge and Hinz (1986) for triose phosphate/phosphate translocator (TPT) and recently by Tripp *et al*. (2007) for ARC6. Apart from inner envelope membrane proteins containing a cleavable transit peptide there are two characterized exceptions. Tic32 (Nada and Soll, 2004) and a quinone oxidoreductase homologue (ceQORH; Miras *et al*., 2002) lack cleavable presequences. Import analysis of Tic32 deletion mutants revealed the importance of the N-terminal part of the sequence in targeting to the inner envelope (Nada and Soll, 2004). In the case of ceQORH, site-directed-mutagenesis and import experiments *in vitro* and *in vivo* revealed that the whole protein sequence consists of two lipid-interacting domains separated by a soluble domain that act concertedly in regulating import. This soluble domain is essential for faithful plastid targeting (Miras *et al.*, 2007).

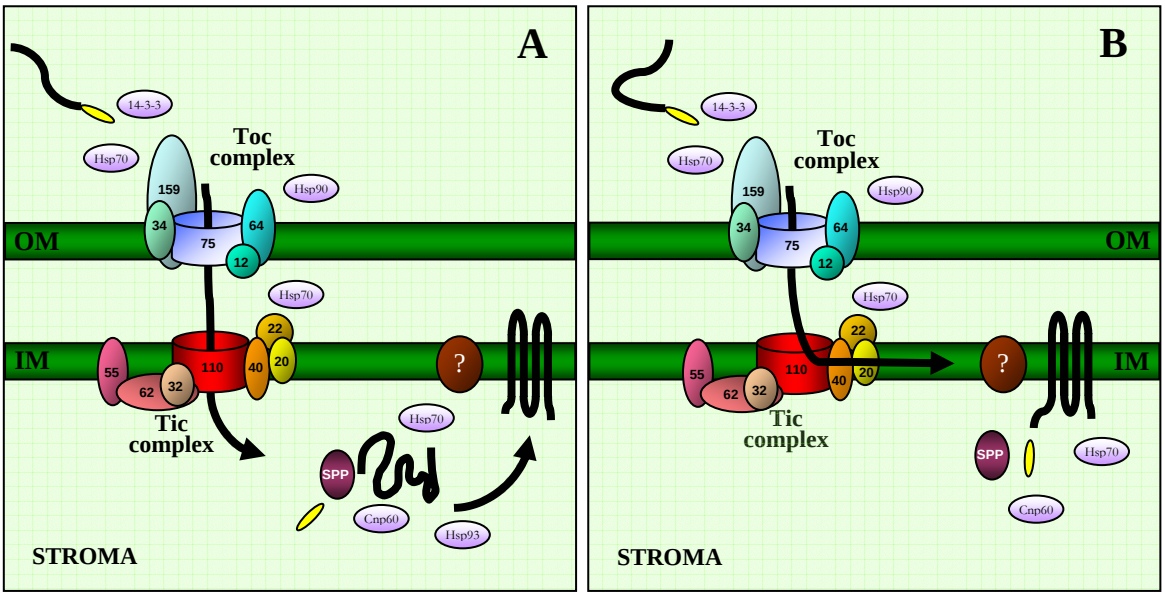


Figure 1. Sorting pathways of chloroplast inner envelope membrane proteins

Proteins that are destined for the inner envelope membrane of chloroplasts use at least two different pathways. **A**. The first pathway is called "conservative sorting". Proteins are transported across the outer and inner envelope membranes into the stroma where they are processed by the stromal processing peptidase (SPP) and reach the inner envelope membrane in an export-like insertion reaction. **B** The second pathway is called "stop-transfer". Precursor proteins are arrested at the level of the inner envelope membrane and are laterally released into the membrane. It remains unrevealed if some other proteinaceous components (?) are involved in the inner envelope membrane insertion. OM, IM indicate the outer and inner envelope membrane, respectively, the numbers indicate the molecular masses of the subunits of the Toc and Tic complexes. The import pathways indicated by arrowheads are shown schematically, therefore the subunits of the import machineries which are crossed by arrowheads do not have to be involved in the import process.

1.3 Import regulation at the level of the Toc complex and in the intermembrane space

The first step of preprotein import into chloroplasts is the recognition by the Toc receptors at the chloroplast surface. This step is regulated by the GTP/GDP cycle (Fulgosi and Soll, 2002; Becker *et al.*, 2004a) of two GTP-binding receptors, namely Toc34 and Toc159. Toc34 in its GTP-loaded state recognizes the C-terminal part of the transit peptide (Becker *et al.*, 2004a). The binding of preprotein to Toc34 stimulates hydrolysis of GTP to GDP (Sveshnikova *et al.*, 2000; Jelic *et al.*, 2002). The Toc34 receptor exhibits low affinity for the transit peptide in a GDP-bound state and therefore releases the preprotein, which is subsequently transferred to Toc159 (Becker *et al.*, 2004a). Toc159 is

characterized as a GTP-driven motor. Hydrolysis of GTP by Toc159 drives the preprotein across the outer envelope membrane through the translocation channel, Toc75 (Schleiff *et al*., 2003b). Toc75, a β-barrel-type protein (Hinnah *et al.*, 1997) cannot mediate translocation of preproteins on its own but relies on the co-ordinate activities of the Toc GTPases and a Hsp70-type chaperone located in the intermembrane space to catalyze transport (Schleiff *et al*., 2003b). Both Toc34 and Toc159 receptors seem to be necessary for import of precursor proteins using the general import pathway. Their inactivation by proteases (Cline *et al*., 1984) inhibits preprotein binding to the organellar surface and subsequently its transport across the chloroplast envelope membranes.

Recently Qbadou *et al.* (2006) described the function of a third outer envelope receptor, Toc64. Toc64 is dynamically associated with the Toc core complex (Toc34, Toc75, Toc159) in pea (Schleiff *et al*., 2003a) and its sequence contains three tetratricopeptide repeats (TPR) (Sohrt and Soll, 2000). The TPR domains are exposed to the cytosol and interact with the cytosolic Hsp90 chaperone which is carrying a preprotein. Dissociation of the preprotein from the chaperone initiates its recognition by Toc34. Finally, delivery of the preprotein from Toc64 to the Toc core complex leads to the dissociation of Toc64 (Qbadou *et al*., 2006). On the *trans* side of the outer envelope membrane Toc64 interacts with intermembrane space Hsp70 (Schnell *et al*., 1994), Toc12 (Becker *et al*., 2004b) and Tic22 (Kouranov *et al*., 1998), facilitating preprotein translocation toward the Tic translocon (Qbadou *et al*., 2007).

1.4 Import regulation at the level of the Tic complex

Protein translocation mechanisms across the inner envelope membrane are not as well characterized as import across the outer envelope membrane. However, two mechanisms are described that may regulate the protein translocation via the Tic machinery. The first import mechanism is regulated by Ca^{2+} and calmodulin (Chigri *et al*., 2005; 2006). The second one depends on the redox state of the chloroplasts and involves three proteins of the Tic complex: Tic32 (Hörmann *et al*., 2004), Tic55 (Caliebe *et al*., 1997) and Tic62 (Küchler *et al*., 2002). Both mechanisms partially overlap since the Tic32 protein was characterized as a predominant calmodulin-binding protein at the inner envelope membrane of chloroplasts (Chigri *et al*., 2006). Tic32 displays homology to conserved short-chain dehydrogenases/reductases which common feature is an NAD(P)H-

binding domain required for their catalytic activity (Hörmann *et al*., 2004). NADPH binding by Tic32 affects its interaction with other Tic components like Tic110 and Tic62, whereas binding of calmodulin to Tic32 promotes its interaction with the translocation channel, Tic110 (Chigri *et al*., 2006). According to this hypothesis import of different proteins could be regulated by association/dissociation of Tic32 from Tic110 that might have some influence on substrate specificity or activity of the translocon.

Recently Stengel *et al*. (2008) described dehydrogenase activity for Tic62 and redox-regulated interaction of this protein with the Tic complex and ferredoxin-NAD(P) oxido-reductase (FNR). Tic62 seems to function as a sensor of the redox status of the chloroplasts by binding FNR at its C-terminus and NADP at the N-terminus. Stengel *et al*. (2008) proposed the redox-regulated shuttling model of Tic62, according to which a major fraction of this protein was found either in the membrane or in the stromal fraction of the chloroplasts. FNR seems to be able to reduce the NADP molecule associated with Tic62 decreasing the interaction of Tic62 with Tic110. Under reduced conditions Tic62 is transferred to the stroma. In contrast, incubation of chloroplasts with oxidizing agents alters the Tic62 distribution within the organelle toward the membrane fraction and promotes binding of Tic62 to the Tic translocon. The NADP/NADPH ratio in the chloroplast could mediate the redox regulation of protein import into chloroplasts, by assembly and disassembly of the translocation complex.

The third protein involved in redox regulation is Tic55. It comprises a Rieske-type iron-sulphur centre and a mononuclear iron-binding site. The oxidation state of the iron atoms could function as a biosensor for the import competence of the chloroplast. Caliebe *et al*. (1997) showed that the modification of histidine residues of the Rieske iron-sulfur cluster resulted in an arrest of preprotein at the level of the inner envelope membrane. This observation, together with fact that Tic55 forms a stable complex with Tic110 and a trapped precursor, suggests a functional role of Tic55 in preprotein translocation.

1.5 Soluble stromal factors, protein processing and folding

Several soluble factors in the stroma are involved in the processing of precursor proteins. As mentioned above, preproteins are processed by the stromal processing peptidase (SPP). The N-terminus of SPP carries an HXXEH zinc-binding motif characteristic for the pitrilysin metalloendopeptidase family, which includes the

mitochondrial processing peptidase of the mitochondrial matrix (VanderVere *et al*., 1995; Richter and Lamppa, 1998; 1999). The SPP interacts directly with 10-15 residues at the C-terminus of the transit peptide, where generally the basic residues are concentrated (Richter and Lamppa, 2002). Cleavage of the transit peptide by SPP leads to immediate release of the mature protein, whereas the transit peptide remains bound to the enzyme (Richter and Lamppa, 1999). SPP then carries out a second processing reaction and converts the transit peptide to sub-fragments. The sub-fragments become a target for rapid ATP-dependent degradation by a second unidentified metallopeptidase in the stroma (Richter and Lamppa, 1999; Moberg *et al*., 2003).

Stromal factors that interact with imported proteins include diverse members of the chaperone family. Hsp93, a chloroplast homologue of Hsp100, also called ClpC (Akita *et al*., 1997; Nielsen *et al*., 1997), functions together with Tic40 and Tic110 in protein translocation across the chloroplast inner envelope membrane into the stroma. Tic40 plays a role as an ATPase activation protein for ClpC (Chou *et al*., 2006). Recently Vojta *et al*. (2007) showed that ClpC seems to be involved in the re-export pathway of the intermediate form of Tic110 from the stroma to the inner envelope membrane of chloroplasts. Other stromal chaperones, which interact with some precursor proteins upon their import, are homologues of Hsp70 and Cpn60 (Lubben *et al*., 1989; Marshall *et al*., 1990; Tsugeki and Nishimura, 1993). It was postulated that they bind precursor proteins to prevent aggregation and participate in correct folding of stromal proteins.

2. Aim of this work

Aim of this work was the characterization of import pathways of the inner envelope membranes proteins. For this purpose several proteins were selected from the literature (Ferro *et al*., 2002; Ferro *et al*. 2003; Froehlich *et al*., 2003; Roland *et al*., 2003 and Eicks *et al*., 2002) on the basis of following criteria:

- Inner envelope membrane localization
- Predicted transit peptide
- Up to nine transmembrane domains

Because import of most characterized inner envelope membrane proteins is mediated by the general import pathway we asked whether import properties of proteins selected for this study would reveal features which distinguish their import pathways from each other and from the import pathways of other known proteins (*e.g*. pSSU). Usually, protein transport is described by energy dependency, by the time necessary to reach the inner envelope membrane, involvement of components of the Toc and Tic complexes and the chloroplast compartment in which the processing takes place. Comparison of these parameters within a selected group of proteins allowed us to notice similarities and differences in their import behaviour. Moreover, we took into consideration two import pathways, which were previously described for mitochondrial inner membrane proteins, the “conservative sorting” and the “stop-transfer”. These routes involve two different translocases at the inner membrane of mitochondria, which transport different sets of precursor proteins. There are three classes of precursor proteins. The first class consists of some inner membrane proteins which contain a cleavable presequence and are transported to their final localization via soluble translocation intermediates in the matrix. Their “conservative sorting” import route is mediated by Tim 23 translocase. The second class consists of inner membrane proteins containing a cleavable presequence and a single membrane span. Transport of these proteins is mediated by Tim 23 translocase according to the “stop-transfer” mechanism. The third class consists of precursor proteins containing many hydrophobic segments and devoid of a cleavable presequence. They are imported into the inner membrane according to the “stop-transfer” mechanism which involves Tim 22 translocase. Transport of proteins according to the “stop-transfer” pathway is arrested at the level of the inner membrane and they are laterally inserted into the lipid bilayer. Therefore a soluble translocation intermediate can not be observed.

The description and comparison of individual import properties of the nine proteins chosen for this study allowed us to create a general model of the import pathway according to which α-helical hydrophobic proteins are transported into the inner envelope membrane of chloroplasts.

2.1. Facts about the proteins chosen as import substrates

Nine proteins were chosen as "tools" for characterizing the import mechanism. All of them were annotated as inner envelope membrane proteins with a predicted transit peptide (Eicks *et al*., 2002; Ferro *et al.*, 2002; Ferro *et al.*, 2003; Froehlich *et al*, 2003; Rolland *et al.*, 2003). Four of them are hypothetical proteins of unknown function, named according to their molecular mass: HP17, HP28, HP29b and HP34. The function of the other five proteins, HP36, IEP37, PIC1, PPT and XPT has been already annotated.

HP36 is annotated in the ARAMEMNON database (Schwacke *et al*., 2002) as an integral inner envelope membrane protein which is involved in transport of sodium ions. The precursor protein consists of 409 amino acids and contains a predicted 74 amino acids long transit peptide. HP36 possesses nine putative transmembrane domains distributed equally over the sequence.

IEP37 is a 37-kDa inner envelope membrane protein described in 1991 by Dreses-Werringloer *et al*. (1991) as a major constituent of the spinach chloroplast inner envelope membrane. The precursor protein consists of 344 amino acids and possesses at the N-terminus a short transit peptide (46 amino acids, Brink *et al*., 1995) forming an amphiphilic α-helix. The mature protein contains one membrane-spanning segment at its C-terminus that possibly anchors the protein within the envelope membrane. The 37-kDa protein is imported into chloroplasts in an ATP-dependent manner. Sequence analysis of the 37-kDa protein suggested its methyltransferase function (Motohashi *et al*., 2003)

Recently Duy *et al*. (2007) described PIC1 (permease in chloroplasts), a 31-kDa precursor protein (296 amino acids) involved in iron transport in chloroplasts. The same protein was also characterized by Teng *et al*. (2006) as a Tic component. PIC1 contains 4 predicted transmembrane helices and is targeted into chloroplasts by a cleavable, 90 amino acids-long transit peptide (Teng *et al*., 2006).

Among all metabolite translocators at the plastid inner envelope, four phosphate translocators (PT) have been identified: triose phosphate (TPT), phosphoenolpyruvate

(PPT), glucose-6-phosphate (GTP) and xylulose-5-P (XPT). They belong to the triose phosphate translocator family which includes also yet uncharacterized PT homologues from plants and other eukaryotes. The family of triose phosphate translocators belongs to the nucleotide-sugar transporter family (NST), which is part of the drug/metabolite transporter superfamily (Flügge, 1999; Flügge *et al*., 2003; Martinez-Duncker *et al*., 2003).

Two metabolite translocators were chosen to study their import pathways into the chloroplasts inner envelope membrane: PPT and XPT.

In the Arabidopsis genome PPT is encoded by eight genes. Only two genes represent full-length genes, *AtPPT1* (protein- 408 amino acids) and *AtPPT2* (protein- 383 amino acids) (Knappe, 2003; Weber *et al.*, 2005). Protein sequence similarity between all PPTs and TPTs amounts 50 to 95% and is restricted to five regions, four of which are putative membrane spanning regions. According to computational analysis (Schwacke *et al*., 2003) the protein possesses a transit peptide. On the basis of hydrophobicity analysis Fisher *et al*. (1997) claimed that PPT has six membrane-spanning regions. Import of *AtPPT1* into cauliflower bud plastids was ATP dependent and did not occurr into plastids pre-treated with thermolysin.

The XPT protein consists of 417 amino acids with a molecular mass of 45 kDa and is encoded by a single gene. Hydrophobicity distribution analysis of the amino acid sequence (Eicks *et al*., 2002) as well as transmembrane helices prediction (Schwacke *et al*., 2003) suggest that this translocator contains six to eight transmembrane helices. XPT possesses a predicted transit peptide at its N-terminus. The processing site is assumed to be located between amino acids 75 and 85. The mature part of XPT is 35-40% identical to TPTs and PPTs and even about 50% identical to GPT proteins (Eicks *et al*., 2002). In XPT as well as in all four classes of phosphate translocators, the conserved dipeptide Lys-Arg located in the fourth region of high similarity is very likely involved in binding of the substrate (Fischer *et al*., 1994).

3. Materials

3.1 Chemicals and membranes

All chemicals used were purchased from established providers: Applichem (Darmstadt, Germany), Biomol Feinchemikalien GmbH (Hamburg, Germany), Fluka Chemie AG (Buchs, Swiss), Merck AG (Darmstadt, Germany), Roth GmbH & Co. (Karlsruhe, Germany), Serva Feinbiochemica (Heidelberg, Germany) and Sigma Aldrich (Munich, Germany). Ni-NTA Superflow was provided by Qiagen (Hilden, Germany). Nitrocellulose (0.2µm) was purchased from Schleicher and Schüll (Dassel, Germany) and 0.37mm blotting-papers from Macherey & Nagel (Düren, Germany). Fuji film imaging plates were used for imaging analysis. They were provided by Fuji photo film company, Japan.

3.2 Kits

For small scale plasmid DNA isolation from bacteria FastPlasmidTM Mini (Eppendorf) or alkaline lysis was used. Large scale DNA isolation was performed using 'Nucleobond AX` supplied by Macherey-Nagel (Düren, Germany). Purification of DNA fragments from agarose gels and purification of PCR products were carried out using Nucleospin Extract II (Macherey-Nagel). DNA sequencing was carried out using BigDye Terminator Cycle Sequencing Ready Reaction Kit supplied by Perkin Elmer (Weiterstadt, Germany). *In vitro* transcription was performed using chemicals from MBI Fermentas whereas *in vitro* translation was done with Wheat Germ Extract Translation Kit supplied by Promega (Madison, USA).

3.3 Molecular weight and size markers

Molecular weight standard MW-SDS-70 from Sigma was used for SDS-PAGE. DNA fragments size marker for agarose gels was prepared by PstI restriction of λ–phage DNA, provided by MBI Fermentas.

3.4 Enzymes

Restriction enzymes, DNA- and RNA polymerase and other nucleic acids modifying enzymes were supplied by Roche (Mannheim, Germany), MBI Fermentas (St. Leon-Rot, Germany), Pharmacia Biotech (Freiburg, Germany) and Sigma. T4-DNA ligase was purchased from Eppendorf (Hamburg, Germany). RNase was supplied by Amersham Pharmacia Biotech, thermolysin by Calbiochem and lysozyme from Serva.

3.5 Oligonucleotides

Oligonucleotides used in PCR reactions were ordered from Invitrogen (Karlsruhe, Germany), MWG Biotech (Ebersberg, Germany) or Qiagen (Hilden, Germany).

3.5.1 For cloning of HP17 into pSP65 vector

HP17_Fwd 5´-ACGC **GTCGAC** ATG GCG TCT CTT TCT TCT ACC-3´
SalI

HP17_Rvs 5´-AAAA **CTGCAG** ATT CAA TTT CAA ATC CTA AAA ACA G-3´
PstI

3.5.2 For cloning of HP29b into pSP65 vector

HP29b_Fwd 5´-ACGC **GTCGAC** ATG GCG ACC ACA CTT CAT TG-3´
SalI

HP29b_Rvs 5´-AAAA **CTGCAG** CTA TGG CCA TTT AGT GAA CTC-3´
PstI

3.5.3 For cloning of HP28 into pSP65 vector

HP28_Fwd 5´-ACGC **GTCGAC** ATG AAT GCG TCC GGC TTA ACT-3´
SalI

HP28_Rvs 5´-AAAA **CTGCAG** AAA AGA CGG GGA AAA GAA AAA AG-3´
PstI

3.5.4 For cloning of HP36 into pSP65 vector

HP36_Fwd 5´-CCG **GAATTC** ATG GCT TCC ATT TCC AGA ATC-3´
EcoRI

HP36_Rvs 5´-ACGC **GTCGAC** TTA CTC TTT GAA GTC ATC CTT G-3´
SalI

3.5.5 For cloning of IEP37 into pSP65 vector

IEP37_Fwd 5´-CCG **GAATTC** ATG GCC TCT TTG ATG CTC AAC-3´
EcoRI

IEP37_Rvs 5´-ACGC **GTCGAC** TCA GAT GGG TTG GTC TTT GGG-3´
SalI

3.5.6 For cloning of HP17 from pSP65 into pET21d vector

HP17_pET21d_Fwd 5´-CATG **CCATGG** TGG CGT CTC TTT CTT CTA C-3´
NcoI

HP17_pET21d_Rvs 5´-CCG **CTCGAG** TCA GGC TGT AGC CTC G-3´
XhoI

3.5.7 For cloning of mOE33 from pOE33/pET21c into pET21d vector

mOE33_Fwd 5´-CTAG **CCATGG** AA GGT GCT CCA AAG AG-3´
NcoI

mOE33_Rvs 5´-GGTG **CTCGAG** TTC AAG C-3´
XhoI

3.6 Vectors

Table 1. Overexpression and translation vectors used in this study

	Overexpression vectors		**Translation vectors**		
Vector name	pET-21c(+)	pET-21d(+)	pSP64	pSP65	pGEM4Z
Company	Novagen, Madison, USA	Novagen, Madison, USA	Promega	Promega	Promega
Reference	Studier and Moffat, 1986	Studier and Moffat, 1986	Melton *et al*., 1984	Melton *et al*., 1984	Yanisch-Perron *et al*., 1985

3.7 Clones

3.7.1 HP17, HP28, HP29b, HP34, PIC1, PPT and XPT

The *Arabidopsis* cDNA of seven proteins used in this study: HP17 (At1g42960), HP28 (At3g51140), HP29b (At3g61870), HP34 (At1g78620), PIC1 (At2g15290), PPT (At5g33320) and XPT (At5g17630) were purchased from Salk Institute Genomic Analysis Laboratory as pUNI51 clones U09946, U09805, U14017, U09750, U18531, U10309 and U12352, respectively. For *in vitro* transcription PIC1 was EcoRI/NotI subcloned from pUNI51 into pGEM 4-Z (Promega, Mannheim, Germany). HP17 and HP29b were EcoRI/SalI subcloned into pSP65 vector, whereas HP28, HP34, PPT and XPT were SalI/PstI subcloned into the same vector (Promega, Mannheim, Germany).

3.7.2 HP36 and IEP37

The HP36 (At2g26900) and IEP37 (At3g63410) cDNA clones from *Arabidposis* were obtained from RIKEN Bioresource Center (Japan) and subcloned EcoRI/SalI into pSP65 vector.

3.7.3 pSSU

Precursor of the small subunit of RubisCO (pSSU) was provided by Prof. Dr. Jürgen Soll and was used for many experiments as a control protein. The total length of pSSU is 573 bp that encode a 21 kDa protein. The presequence is 165 bp long and originates from soybean *Glycine max* whereas mature part is from *Pisum sativum* (Lubben and Keegstra, 1986). The sequence was cloned into pSP64 vector.

3.7.4 Tic32

The coding region of Tic32 from pea encoding for 32 kDa protein was cloned into the pSP65 vector. The clone was kindly provided by Prof. Dr. Jürgen Soll and precursor protein was used as a control in the import experiment with chloroplasts treated with Ophiobolin A (section 4.3.3).

3.7.5 tpSSU-110N-mSSU

The hybrid construct tpSSU-110N-mSSU (Lübeck *et al*., 1997) is 1257 bp long and encodes the 46 kDa protein. It was used as a control protein in an experiment of chloroplast fractionation into a soluble and insoluble fraction. tpSSU-110N-mSSU contains the presequence of pSSU (1-192 bp), the N-terminal part of Tic110 (112-817 bp) and the mature part of SSU (175-564 bp) pSSU sequence originates from *Nicotiana sylvestris* (locus NSRUBSSU, Acc. No. X53426, Jamet *et al*., 1991) whereas Tic110 sequence originates from *Pisum sativum*. tpSSU-110N-mSSU was cloned into pET21d vector.

3.7.6 pOE33 and mOE33

Oxygen evolving complex protein of 33 kDa (pOE33) and its mature form (mOE33) were used for import competition experiments (sequence published by Murata *et al.*, 1987). pOE33 was cloned into pET21d vector and consist of 900 bp whereas mOE33 was cloned into pET21c vector and is 747 bp long.

3.8 Bacterial strains

For amplification of DNA, *Escherichia coli* strain DH5α (GibcoBRL, Eggenstein, Germany) was used whereas for overexpression of proteins, BL21 (DE3) (Novagen, Madison, USA) celles were used.

3.9 Growth media

LB or M_9ZB media were used for overexpression or growth of transformed *E. coli* strains for cloning.

LB medium (components/liter)

NaCl	10 g
Peptone	10 g
Yeast	5 g
Agar (for plates)	15 g

M9ZB medium (components/liter)

NH_4Cl	1 g
NaH_2PO_4	3 g
Na_2HPO_4	6 g
Tryptone	10 g
NaCl	5 g

3.10 Radioisotopes

[^{35}S] Methionine/Cysteine mixture with specific activity of 1000 Ci/mM was provided from Amersham Biosciences (Freiburg, Germany).

3.11 Plant material

Pea (*Pisum sativum*) of the sort “Arvika” (Praha, Czech Republic) was grown on vermiculite or on sand under 12 h day / 12 h night cycle in a climate chamber at 20°C.

4. Methods

4.1 General molecular biology methods

4.1.1 Standard methods

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

4.1.2 Plasmid DNA isolation

Isolation of plasmid DNA from 5ml culture for restriction analysis, subcloning, re-transformation into another bacterial strains and sequence analysis were all adapted from the methods of Holmes and Quigley (1981). Large amount of DNA for *in vitro* transcription and translation was isolated from 100 ml bacterial cultures by NucleobondAX kit supplied by Machery-Nagel following manufacturer´s instructions. Fast purification of restricted plasmid DNA was performed by QiaQuick PCR Purification Kit from Qiagen or Nucleospin Extract II Kit from Macherey-Nagel.

4.1.3 Polymerase chain reaction (PCR)

The restriction sites for cloning of DNA fragments into plasmid vectors were added by the polymerase chain reaction (Saiki *et al*., 1998). The standard PCR reactions included 100ng DNA template, 200µM of each dNTPs, 200nM of primers and 1-2 units of taq polymerase in the supplied buffer (TripleMaster PCR System, Eppendorf, Hamburg, Germany). Temperatures were adjusted corresponding to the annealing temperatures of the primers. Prior to ligation with corresponding inserts, vectors were dephosphorylated by use of alkaline phosphatase from calf intestine (Roche Diagnostics GmbH, Mannheim, Germany).

4.1.4 Cloning techniques

Isolation and restriction of plasmid DNA and PCR-amplified fragments as well as ligation and agarose gel electrophoresis were performed according to standard procedures (Sambrook *et al.*, 1989). The reaction conditions for the enzymes were adjusted according to the protocols provided by the manufacturers. Standard techniques were applied for the ligation of all clones used in these studies into both pSP65 and pET21 vectors.

4.1.5 *In vitro* transcription and translation

Prior to *in vitro* transcription, plasmids containing clones listed in section 3.7, were linearized for 60 minutes at 37°C with restriction enzymes listed in the tables below (Table 2).

Table 2. Restriction enzymes used for the linearization of clones used in this study

	HP17	**HP28**	**HP29b**	**HP34**	**FD3C**	**PIC1**
Restriction enzyme	PstI	SalI	PstI	SalI	PstI	NotI

	PPT	**XPT**	**HP36**	**IEP37**	**pSSU**	**Tic32**
Restriction enzyme	SalI	SalI	SalI	SalI	EcoRI	PstI

Plasmid DNA was isolated and purified using phenol-chlorophorm purification or QiaQuick PCR Purification Kit from Qiagen. The final pellet was resuspended in RNase free water or 0,1% TAE buffer. *In vitro* transcription of linearized plasmids was carried out in a reaction volume of 50 µl containing transcription buffer (supplied by MBI Fermentas), 10mM DTT, 100U RNase inhibitor, 0.05% (w/v) BSA, 0.5mM ATP, CTP, and UTP, 0.375 mM m^7 -Guanosin (5') ppp (5') Guanosine (cap), 10U SP6 RNA polymerase and 2-3 µg linearized plasmid DNA. The reaction mixture was incubated for 30 minutes at 37˚C to yield RNA with cap at the 5'-end. Finally, 1.2 mM GTP was added and the transcription mixture was incubated for another two hours. mRNA was either used directly for *in vitro* translation or stored under liquid N_2. *In vitro* translation of mRNA was carried out using

the Wheat Germ Lysate System, following the manufacturer's instructions, with optimal RNA concentration and adjusted potassium acetate concentrations, which were determined by test translation. 150µCi of [^{35}S]-methionine/cysteine mixture were added for radioactive labelling. After translation the reaction mixture was centrifuged at 50,000xg for 20 minutes at 4°C and the supernatant was used for import experiments.

4.2 Isolation of intact chloroplasts from pea

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

4.3 Treatment of chloroplasts and translation product before import

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

Before chloroplasts isolation, the peas were left over night in the dark. After the isolation procedure, intact chloroplasts were left on ice in the dark for 30 minutes in order to deplete ATP and therefore allow subsequent import experiments to be carried out with

only exogenously added ATP as energy source. To deplete endogenous ATP from *in vitro* translation product, Micro Bio-Spin Chromatography Columns (Bio-Rad, Hercules, CA, USA) were used according to manufacturer´s recommendations.

4.3.2 Protease pre-treatment of isolated intact chloroplasts

Protease treatment of chloroplasts before insertion of radioactively labelled protein was carried out using chloroplasts corresponding to 1 mg chlorophyll, 1 mg thermolysin and 0.5 mM $CaCl_2$. Wash medium (330 mM sorbitol, 50 mM HEPES/KOH, pH 7,6, 3 mM MgCl2) was added to the final volume of 1 ml and the sample was incubated for 30 minutes on ice. To stop the protease reaction, 5 mM EDTA was added. Intact chloroplasts were re-isolated via a discontinuous Percoll gradient containing 5 mM EDTA and washed twice as described before.

4.3.3 Chloroplasts pre-treatment with Ophiobolin A and ionophore A23187

Intact pea chloroplasts were treated with specific chemicals in order to block import of pre-proteins at the level of the inner membrane. Prior to the import reaction described in section 4.4.1 chloroplasts were incubated with 100 µM Ophiobolin A for 20 minutes at 25°C or with 50 µM A23187 for 20 minutes at 4°C.

4.4 Import experiments and chloroplasts post-treatment

4.4.1 Import of radioactively labelled proteins into intact chloroplasts

^{35}S-labelled precursor proteins (translation products) in maximal amounts of 10% (w/v) in the reaction were mixed with freshly prepared intact pea chloroplasts (equivalent to 15-20 µg chlorophyll) in import buffer (330 mM sorbitol, 50 mM HEPES/KOH pH7.6, 3 mM $MgSO_4$, 10 mM methionine, 10 mM cysteine, 20 mM K-gluconate, 10 mM $NaHCO_3$, 2% BSA (w/v) and up to 3 mM ATP) in a final volume of 100 µl (Waegemann and Soll, 1995). The import mix was incubated at 25˚C for 1 to 32 minutes according to experimental requirements. Chloroplasts were re-isolated over 40% Percoll cushions, washed twice and the samples were separated by SDS-PAGE. The resulting gels were coomassie-stained, dried and exposed on X-ray sensitive screens over night.

4.4.2 Chloroplasts post-treatment with thermolysin

To control the efficiency of protein import across the outer envelope of chloroplasts the intact organelles were treated with the protease thermolysin. After import chloroplasts were pelleted at 1500xg for 1 minute at 4°C and resuspended in 100 µl digestion buffer (330 mM sorbitol, 50 mM HEPES/KOH pH 7.6, 0.5 mM $CaCl_2$). The addition of thermolysin (0.5 µg per 1 µg of chlorophyll) started the digestion which was performed for 20 minutes on ice. The reaction was stopped by addition of 5 mM EDTA. Chloroplasts were pelleted and washed in the washing buffer (330 mM sorbitol, 50 mM HEPES/KOH pH 7.6, 5 mM EDTA).

4.4.3 ATP concentration scale

To characterize ATP requirements of proteins imported into isolated intact chloroplasts, radioactively labelled, ATP-depleted translation product was incubated with chloroplasts corresponding to 20 µg chlorophyll in the import mixture (see section 4.4.1) without or with different concentration of ATP: 10, 25, 75, 200, 1000 and 3000 µM. Samples were incubated at 25˚C for 8 minutes. Chloroplasts were re-isolated over 40% Percoll cushions and subsequently samples were analyzed by SDS-PAGE. All steps were performed in the dark to minimize the generation of internal ATP via photophosphorylation.

4.4.4 Pulse-chase import experiment

To observe the changes in localization and quantity of imported protein during the time radioactively labelled precursor proteins were incubated for 3 minutes on ice with isolated chloroplasts corresponding to 20 µg chlorophyll in import mixture without ATP (see section 4.4.1). These conditions enable binding of the precursor protein to the receptors at the chloroplasts surface but not import into the organelle (pulse). Chloroplasts were pelleted at 1500xg for one minute, washed once in the import buffer and the final pellet was resuspended in import buffer containing 3 mM ATP (chase) to allow complete import. The import reactions were performed from 0 to 32 minutes at 25°C. Import was stopped after different times by addition of Laemmli buffer and samples were analyzed by SDS-PAGE. The experiment was performed in the dark to minimize the generation of internal ATP produced via photophosphorylation.

4.4.5 Competition for import with mOE33 and pOE33

5 μM of overexpressed and purified competitor pOE33 and its mature form mOE33 were added to the import reaction. The import experiment was performed as described in section 4.4.1 with some changes: up to 15 μg of chlorophyll were used and the import reaction lasted 5 minutes for pSSU and 10 minutes for all other proteins used in this study.

4.5 Suborganellar localization of imported proteins

4.5.1 Fractionation of chloroplasts into soluble and membrane fractions after import

To distinguish between integral membrane proteins and soluble proteins, chloroplasts were lysed after import in 10 mM HEPES/KOH pH 7.6 for 20 minutes on ice. Subsequent centrifugation at 256,000×g for 10 minutes at 4°C separated the membranes from a soluble fraction. Both fractions were analyzed by SDS-PAGE.

4.5.2 Extraction of proteins with 6 M urea

After import chloroplasts were re-isolated over 40% Percoll cushions, lysed as described in the section 4.5.1 and pelleted at 256,000xg for 10 minutes at 4°C. The pellet was subsequently treated with 6 M urea in 10 mM HEPES/KOH pH 7.6 for 10 minutes at room temperature (RT). Samples were centrifuged at 256,000xg for 10 minutes at RT and the pellet and soluble fraction were analyzed by SDS-PAGE.

4.6 Stromal processing assay

Intact chloroplasts were isolated as described in the section 4.2. Chloroplasts corresponding to 800 μg chlorophyll were pelleted at 1500xg for 1 minute at 4°C and lysed in 1 ml of 5 mM ice-cold HEPES/KOH pH 8.0 for 15 minutes on ice. Samples were centrifuged for 10 minutes at 16,000xg at 4°C and the supernatant was centrifuged again for 30 minutes at 137,000xg, 4°C. In the processing assay the supernatant containing an active stromal processing peptidase was used. Samples containing 15 μl of supernatant, 2.5 μg chloramphenicol, 20 mM HEPES/KOH pH 8.0, and 4-8 μl radioactively labeled

translation product were mixed in a total volume of 25 µl and incubated for 90 minutes at 26°C. The reaction was stopped by addition of Laemmli buffer and samples were analysed by SDS-PAGE.

4.7 Overexpression and purification of pOE33 and mOE33

Transformed BL21 (DE3) competent cells were grown in 100 ml LB medium containing 100 µg/ml ampicilin, 1 mM $MgSO_4$ and 0.4% glucose till the OD_{600} reached 0.6. Expression was induced by 1 mM IPTG and cells were grown for 3 hours at 37°C. Cultures were centrifuged for 20 minutes at 4000xg at 4°C. Expressed proteins containing a His-tag were isolated in form of inclusion bodies and purified under denaturing conditions according to QIAgen protocols. The protein of interest was eluted by acidic pH. Refolding of the protein was accomplished by dialysis against 6 (over night), 4 (four hours), 2 (four hours) and 0 M urea. Aggregated (misfolded) material was pelleted by centrifugation at 27,000xg for 10 minutes at 4°C. The protein concentration in the supernatant was estimated and used for competition experiments.

4.8 Methods for separation and identification of proteins

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

Electrophoretic separation of proteins under denaturing conditions was performed in discontinuous gel system (Laemmli, 1970). Separation gels varied from 12.5 to 15% acrylamide, whereas for stacking gels 5% acrylamide was used in all cases.

4.8.2 Detection of proteins

After separation of proteins in polyacryamide gels a number of standard detection techniques were used. Staining solution containing 0.18% (w/v) Commassie Brilliant Blue R250 in 50% (v/v) methanol and 7% (v/v) acetic acid enable visualisation of bands equivalent to 0.1- 10µg protein. Gels were stained for 15 minutes on a shaker; unbound dye was removed by 15-30 minutes washing in destaining solution (40% (v/v) methanol, 7% (v/v) acetic acid, 3% (v/v) glycerol). For detection of ^{35}S-labelled proteins, the

acrylamide gels were stained by Coomassie Blue, dried and exposed on imaging plates (BAS-MS) over night. The plates were screened using phosphoimaging scanner FLA-3000 and bands intensities were analyzed using AIDA program for advanced image analysis (Advanced Image Data Analyzer v.3.52, 2D densitometry).

4.8.3 General methods of protein biochemistry

Determination of protein concentration was performed using the Bradford Bio-Rad reagent. For concentrating proteins from diluted solutions, 10% final concentration of TCA (w/v) was added to samples and incubated for 20 minutes on ice, followed by 15 minutes centrifugation at 25,000xg. Samples were neutralized by Tris-base, as demanded by experimental conditions.

5. Results

Proteins used in this thesis were selected from proteomics studies: Ferro *et al*., 2002; 2003; Froehlich *et al*., 2003 and Roland *et al*., 2003 and in the case of XPT from Eicks *et al*., 2002 on the basis of following features:

- Predicted chloroplast inner envelope membrane localization
- Predicted presence of transit peptide
- Up to nine predicted transmembrane domains
- Effective transcription and translation *in vitro*

The number of predicted transmembrane helices was checked using the ARAMEMNON database (Schwacke *et al*., 2003), whereas the length of a predicted transit peptide was checked using the ChloroP program (Emmanuelson *et al*., 1999).

Characteristics of all chosen proteins are shown in the tables below (Table 3).

Table 3. Characteristics of proteins used in this study

	HP17	HP28	HP34	HP36	IEP37
Function	unknown function	unknown function	unknown function	putative sodium-dependent bile acid symporter	SAM-dependent methyl transferase
AGI Acc no.	At1g42960	At3g51140	At1g78620	At2g26900	At3g63410
Predicted length of protein precursor (aa)	168	278	342	409	338
Predicted length of transit peptide (aa) according to ChloroP	59	66	65	74	51
Calculated molecular mass (kDa)	17,8	27,9	34,8	36,1	37,9
Predicted transmembrane domains according to Aramemnon	1	4	6	9	1

aa- amino acids

Table 3 cont. Characteristics of proteins used in this study

	XPT	HP29b	PIC1	PPT
Function	Xul-5-P translocator	unknown function	permease in chloroplast	Phosphoenol--pyruvate translocator
AGI Acc no.	At5g17630	At3g61870	At2g15290	At5g33320
Predicted length of protein precursor (aa)	417	272	296	408
Predicted length of transit peptide (aa) according to ChloroP	55	70	15	30
Calculated molecular mass (kDa)	45	29,5	31,2	44,2
Predicted transmembrane domains according to Aramemnon	7-8	4	4	6

aa- amino acids

5.1 Protein import into chloroplasts and post-treatment with thermolysin

In an initial approach import capabilities of nine inner membrane proteins were investigated. The *in vitro* translated, radioactively labelled preproteins (HP17, HP28, HP34, HP36, IEP37, XPT, HP29b, PIC1 and PPT) were depleted of ATP and imported into isolated chloroplasts under standard conditions (see section 4.4.1). Prior to import chloroplasts were kept in the dark to deplete intraorganellar ATP. For each protein two kinds of samples were prepared: without and with externally added ATP. Only in the presence of ATP, preproteins could be imported into the organelle and processed to their mature forms; import reactions of all precursors were ATP dependent. The difference in size between the preproteins and their mature forms corresponds to the predicted transit peptides, although in some cases the predicted length of the transit peptide differs from those observed in experimental approach (compare Table 3 with the results presented below). To confirm that the mature proteins were located inside the chloroplasts, after import half of each sample was treated with thermolysin. Thermolysin digests proteins present outside the organelle but can not penetrate the outer envelope membrane under

applied conditions. Imported proteins were not digested by thermolysin that indicates their localization inside the organelle. In the case of HP17, HP28, HP36, IEP37 and PPT a small amount of precursor proteins was also protease resistant (Fig. 2, A and B, lanes –ATP, +T) suggesting that the thermolysin digestion process was not as efficient as for other precursor proteins used. The proteins can be initially sorted into two groups according to their behavior during import. For the first group (HP17, HP28, HP34, HP36, IEP37 and XPT) processing resulted in one product, the mature form of the protein (m) protected inside the chloroplasts after thermolysin treatment (Fig. 2, A). These proteins contain a transit peptide, which consists of a single part cleaved off by the processing peptidase. Therefore we called them "single" transit peptides in contrast to bipartite transit peptides, which are removed from the protein by a double cleavage. Between the precursor and the mature protein of HP17 (Fig. 2 A, +ATP, +T, asterisk, ∗) an additional band resistant to thermolysin treatment was slightly visible. This band was not considered as an import intermediate because it was not always present in repetitions of this experiment. Moreover, for HP17, the thermolysin digestion seemed to be not efficient since some amount of precursor protein (p) was still present (Fig. 2 A, +ATP, +T). A second possibility could be that the import occurs faster than processing so that the preprotein accumulates. For HP28 without externally added ATP and after thermolysin treatment, a characteristic protein fragment was observed (Fig. 2 A, -ATP, +T, Tim). It represents most likely a translocation intermediate similar to the translocation intermediates (Tims) observed for pSSU (Friedmann and Keegstra, 1989; Waegemann and Soll, 1991).

In the second group (HP29b, PIC1, PPT) preproteins were processed twice resulting in the presence of two protease resistant forms inside the organelle: intermediate (i) and mature protein (m, Fig. 2, B). Similarly as for HP17, an additional band between the intermediate and the mature form of PIC1 was visible (Fig. 2 B, +ATP, +T, asterisk, ∗). This band was protected inside the organelle after thermolysin treatment. Its origin remains unknown, because similarly as in the case of HP17, it was not always present in repetition of this experiment.

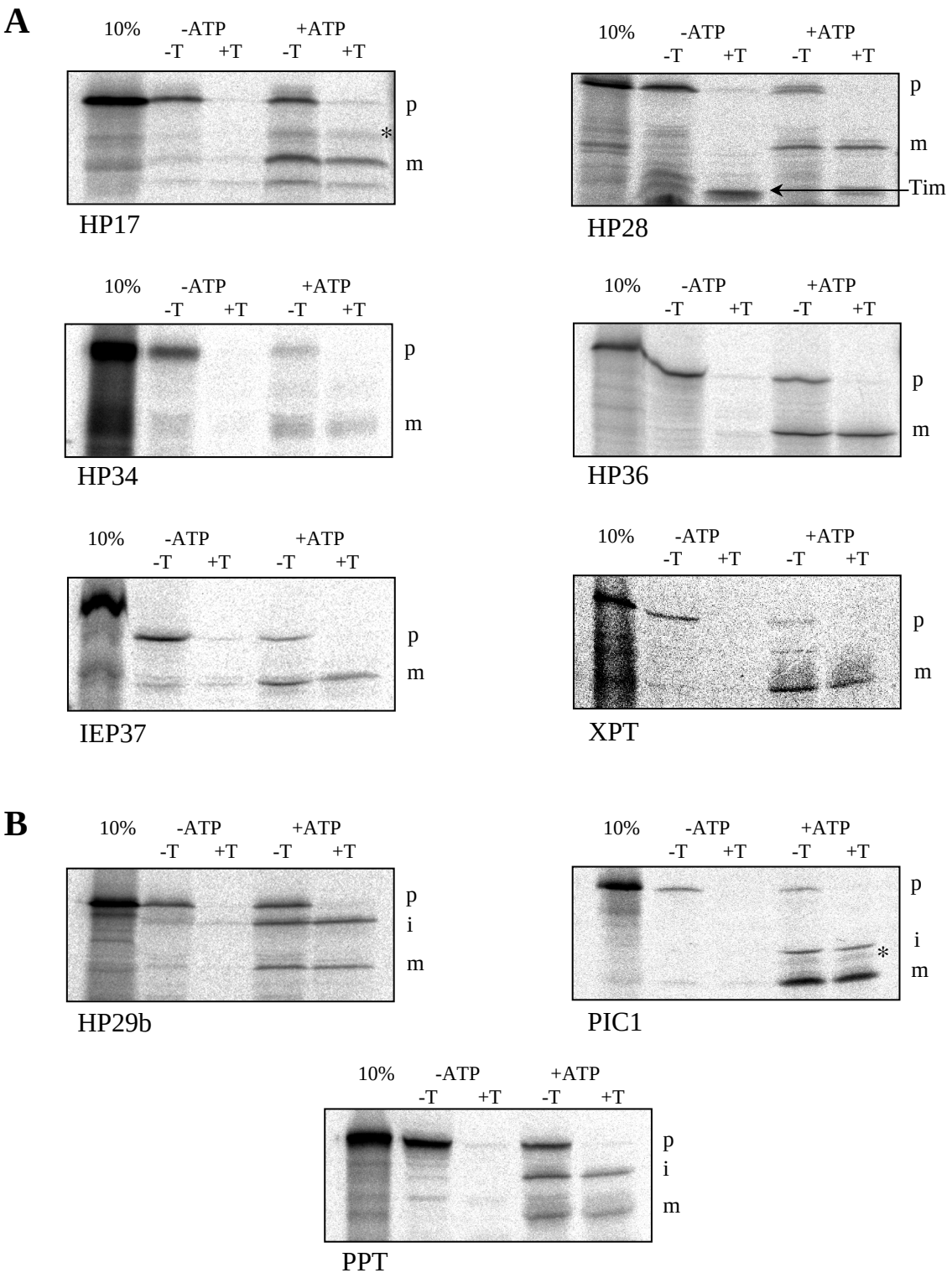


Figure 2. Import of radioactively labelled HP17, HP28, HP34, HP36, IEP37, XPT (A) and HP29b, PIC1, PPT (B) into chloroplasts

Import reactions were performed for 15 minutes at 25°C in the absence (-ATP) or presence of 3 mM ATP (+ATP). After import chloroplasts were reisolated on a Percoll cushion, washed and not treated (-T) or treated with thermolysin (+T). Preproteins (p) not imported into the chloroplasts were digested by thermolysin, whereas mature forms of the proteins (m) in **A** and both intermediates (i) and mature forms (m) in **B** were protected inside the organelle. Samples were analyzed by SDS-PAGE. The first lane (10%) represents 1/10 of the translation products used for the import reaction. Asterisk (∗) represents bands of unknown origin, Tim represents a translocation intermediate.

5.2 Stromal processing assay

The initial import experiments revealed two groups of proteins, which possess either a “single”- or a bipartite transit peptide. Some inner envelope proteins, which are targeted into the organelle by a cleavable transit peptide, are processed in the stroma by the stromal processing peptidase (SPP). To test whether the processing of proteins used in this study is carried out by the SPP we conducted stromal processing assays (Abad *et al.*, 1989). This approach was especially interesting for HP29b, PIC1 and PPT, which were classified to the group of proteins possessing a bipartite transit peptide (section 5.1). We also asked whether SPP is responsible for the double processing, to the intermediate and the mature form of these proteins.

Intact chloroplasts were isolated according to the protocol described in section 4.2 and lysed in 5 mM ice-cold HEPES/KOH pH 8.0 for 15 minutes on ice. A supernatant containing an active stromal processing peptidase was obtained after centrifugation steps described in section 4.6 and used in this experiment. In a total volume of 25 µl, radioactively labeled translation product was incubated at 26°C for 90 minutes with 15 µl of stromal supernatant, 2.5 µg chloramphenicol and 20 mM HEPES/KOH pH 8.0. Under these conditions a control protein, pSSU, was processed to its mature form, mSSU (Fig. 3 A, lane SPP). Similarly, for HP28, HP34, HP36 and IEP37 processing also took place in the stromal extract and mature forms of these four precursor proteins were clearly visible. In the case of HP17 and XPT processing was not as efficient as for the control protein. Whereas for HP17 the mature protein could be detected, for XPT it was almost impossible because of the presence of many other unspecific bands (Fig. 3 A).

Among precursor proteins classified in this work as the group of proteins processed twice (HP29b, PIC1 and PPT), only PIC1 was clearly processed by SPP to its intermediate and mature form (Fig. 3 B). This result is quite surprising taking into consideration processing of other proteins containing a bipartite transit peptide, Toc75 (Inoue *et al.*, 2005) and Tic40 (Tripp *et al.*, 2007). Both of them are processed to their intermediate forms in the stroma but the second processing probably takes place in the intermembrane space.

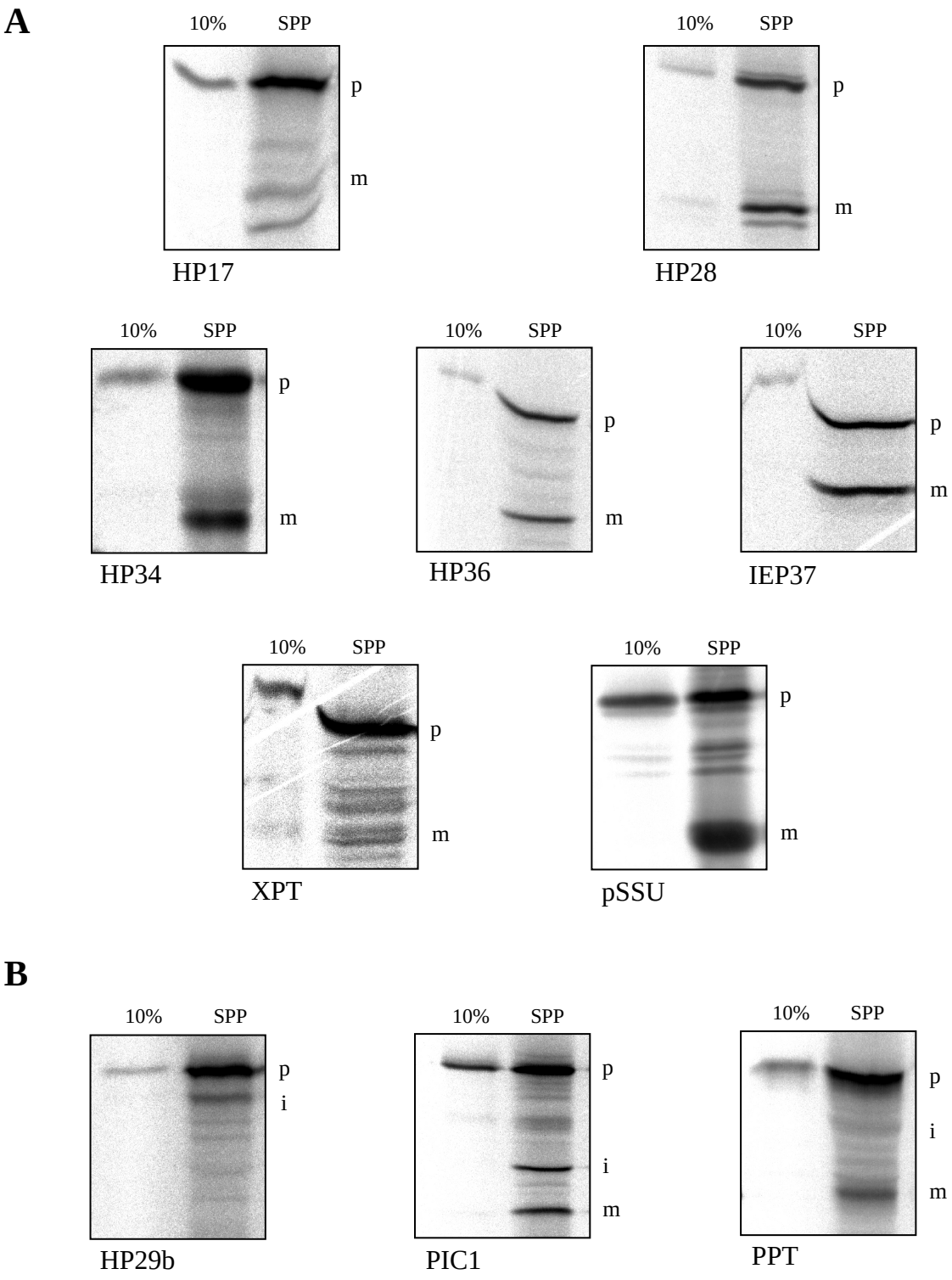


Figure 3. Processing of precursor proteins in the isolated stromal extract
Stromal extract containing an active processing peptidase was incubated with radioactively labelled translation product of HP17, HP28, HP34, HP36, IEP37, XPT, HP29b, PIC1, PPT and pSSU as a control, 2.5µg chloramphenicol and 20 mM HEPES/KOH, pH 8.0 for 90 minutes at 26°C. Precursor proteins (p) of HP17, HP28, HP34, HP36, IEP37 and pSSU were processed either to their mature forms (m) whereas PIC1 was processed by SPP to both the intermediate (i) and mature form (m). XPT processing could not be clearly recognized, similarly as the processing of HP29b to the mature protein and PPT to the intermediate form. 10% indicates 1/10 of a translation product used for the experiment; SPP indicates the stromal processing peptidase present in the stromal extract.

HP29b seems to be processed according to this model. SPP probably processed HP29b to the intermediate but not to the mature form (Fig. 3 B, i). Therefore another protease could be involved in maturation of HP29b. Unfortunately, methods available are not able to isolate only the intermembrane space content and use it similarly to a stromal extract. The question of which protease is involved in the processing of HP29b remains open. In the case of PPT, SPP seems to be involved only in processing of the intermediate into the mature form of the protein (Fig 3 B, m). The processing of the PPT precursor to its intermediate was not quite clear, although among many slightly visible bands, which were considered as a background, one stronger band of the molecular mass similar to intermediate form of PPT was observed (Fig. 3 B, i). Similarly as for HP29b, results observed for PPT suggest involvement of another protease in processing.

The results observed indicate that SPP seems to be involved in generation of all mature forms except from HP29b. It would suggest that at least the N-terminal parts of most proteins reach the stroma at some point during the translocation process.

At this point it should be considered that the experiments represent an *in vitro* system that never exactly reflects the reactions which take place *in vivo*. Although most proteins were processed by SPP in applied conditions, for some of them, *e.g.* XPT, HP29b or PPT the same conditions might not be optimal for processing. Therefore it remains to be investigated whether in the processing of XPT, HP29b and PPT another protease is involved or the conditions of the stromal processing assay have to be adapted.

5.3 Pulse-chase import experiments

Pulse-chase import experiments were performed to characterize import kinetics of proteins used in this study and particularly to test if the import intermediates of HP29b, PIC1 and PPT are on a productive pathway to the mature form (Fig. 2 B).

Radioactively labelled precursor proteins were incubated with intact chloroplasts for three minutes on ice, without external ATP (pulse). Under limiting ATP conditions and low temperature (4°C) preproteins can only bind to the chloroplast surface. Chloroplasts were pelleted, washed and a fresh import mixture containing 3 mM ATP was added. Addition of energy source and changing the temperature for 25°C (chase) was essential for transport into chloroplasts of all precursor proteins classified to the first (Fig. 4 A; proteins

containing a single transit peptide) and the second group (Fig. 4 B; proteins containing a bipartite transit peptide), and their processing to the mature forms.

Already at very short import times (1-2 minutes) preproteins containing a “single” transit peptide were imported and their mature forms observed although, import efficiency of all of them after 32 minutes of reaction was rather low (Fig. 4 A). In most cases after eight minutes import was completed. The import kinetics of HP17 was quite different from import kinetics of other preroteins containing a “single” transit peptide which were used in this experiment. HP17 precursor protein reached the steady-state level of import already after two minutes of import reaction. Interestingly, longer incubation of HP17 under applied import conditions (16-32 minutes) not only did not increase the import rate but even slightly decreased it. It could suggest that during the prolonged import time HP17 degrades.

During import of HP17, HP28, HP36 and XPT additional bands of unknown origin were observed and are marked by asterisk (Fig. 4 A). They were also present in the previously described assay (section 5.1) and were digested by thermolysin, apart from bands observed during import of HP17 (Fig. 2 A). Most probably they represent bands observed also in the translation product (Fig. 4 A, 10%). During the 32 minutes import their intensity did not change, in contrast to the mature forms of the proteins. Therefore the bands marked by asterisk might represent solely the “impurities” of the translation product.

The appropriate reaction conditions (3 mM ATP, 25°C) initiated import of HP29b, PIC1 and PPT into chloroplasts (Fig. 4 B). Two separate processed forms were clearly visible, one earlier than the other. The order of their appearance was in agreement with a stepwise processing of precursor protein to the intermediate form and further to the mature form. The intermediate form of all preproteins classified to the group B (Fig. 4 B) appeared already at the earliest time point and decreased at later time points while simultaneously the mature form increased. It suggests that the bands described as an intermediate form were on a productive pathway to mature forms of HP29b, PIC1 and PPT.

Obtained results support the hypothesis that the proteins presented in the Fig. 4 B might contain a bipartite transit peptide.

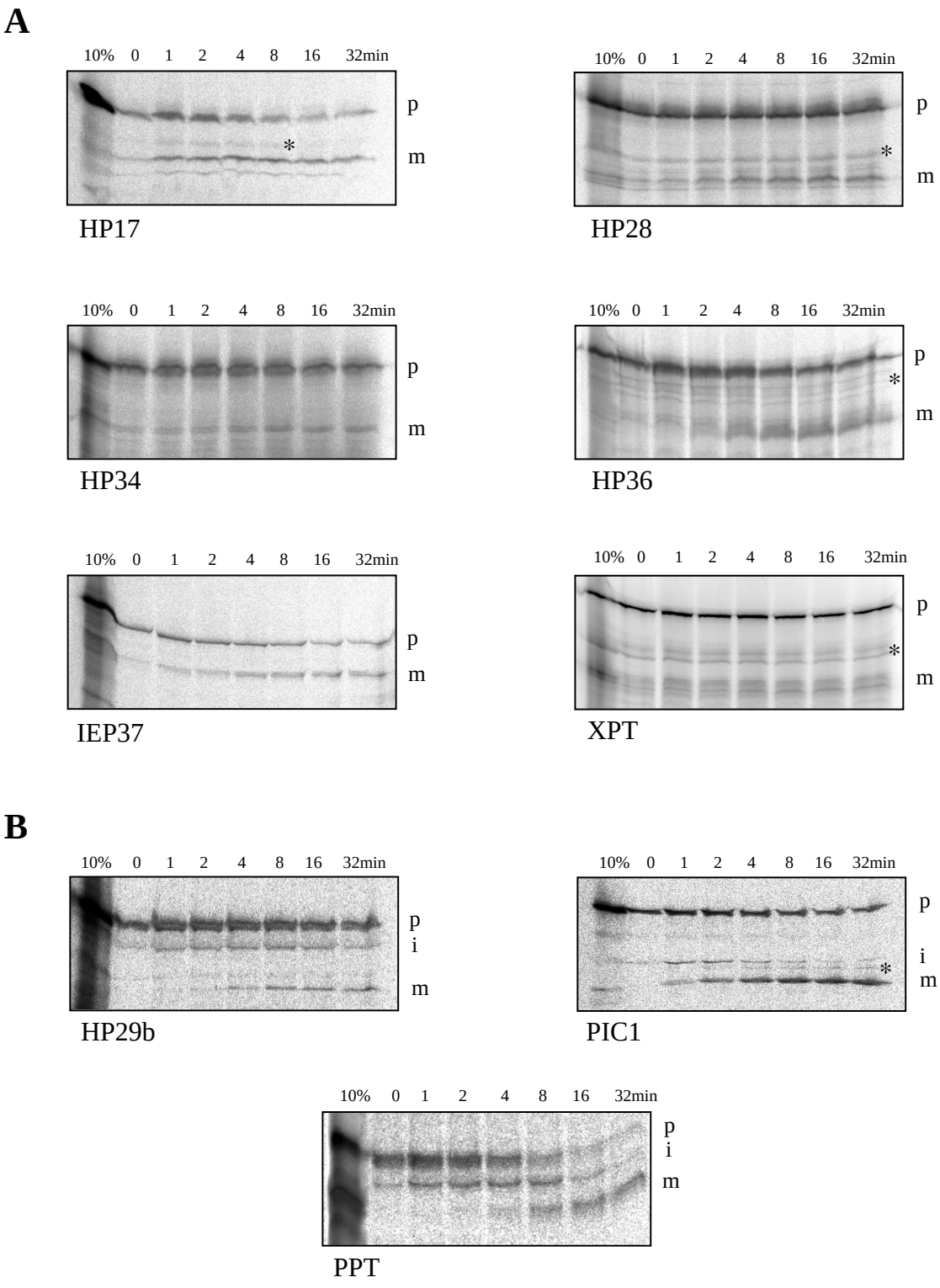


Figure 4. Pulse-chase import experiment of precursor proteins containing a"single" (A) or a bipartite transit peptide (B)

Radioactively labelled precursor proteins (p) were incubated with chloroplasts corresponding to 20 µg chlorophyll on ice for 3 minutes (pulse).The addition of 3 mM ATP and an increase of the temperature to 25°C (chase) enabled import. The import reactions were performed from 0-32 minutes as indicated. Reactions were stopped by addition of Laemmli buffer and the results were analyzed by SDS-PAGE. 10% indicates 1/10 of the translation product used for import experiment, asterisk (∗) indicates either bands which most probably originate from the wheat germ extract containing also the translation product (A) or band of unknown origin (B).

5.4 Energy requirement for import into chloroplasts

ATP is necessary for most of the precursor proteins to be imported into chloroplasts. 50 μM ATP is considered as a "limiting concentration" below that, preproteins using the general import pathway can not be completely translocated into the organelle. Previous experiments (sections 5.1 and 5.3) showed ATP dependency of all proteins used in this study but did not characterize their exact energy requirement.

For the purpose of precise ATP requirement, different ATP concentrations were added to standard import experiments (section 4.4.1). Prior to import, isolated chloroplasts were kept in the dark to deplete internal ATP. ATP was also depleted from all translation products. Import experiments characterizing energy requirements of precursor proteins tested were done in the dark at 4°C. Under these conditions only externally added ATP was supposed to influence their import rates. The import reaction was performed for 8 minutes at 25°C, in the absence or presence of 10, 25, 75, 200, 1000 and 3000 μM ATP. For most precursors investigated, 10 μM ATP was sufficient to observe small amounts of the mature form of the protein (Fig. 5 A and B, lanes 10 μM ATP). With increasing ATP concentrations less precursor protein was bound to the chloroplast surface because more preprotein was imported into the organelle and processed to the mature form (m). For all proteins maximal import rates were observed between 200-1000 μM ATP.

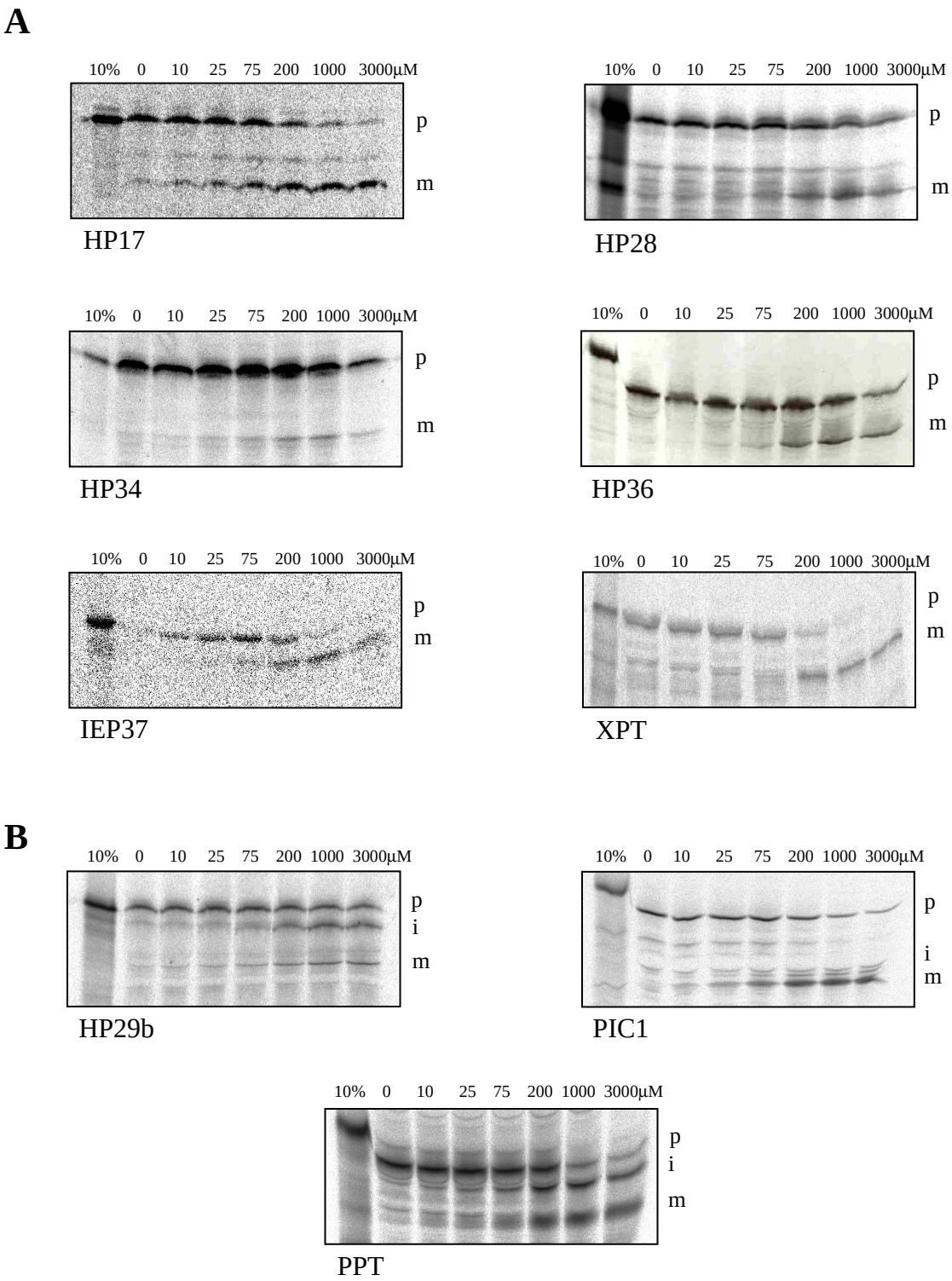


Figure 5. Import with different concentrations of ATP
Import into intact pea chloroplasts was performed by incubating radioactively labelled precursor proteins containing a "single" **(A)** or a bipartite **(B)** transit peptides with chloroplasts corresponding to 20 µg chlorophyll in the standard import mixture. Internal ATP was depleted from both chloroplasts and translation products. Different ATP concentrations were externally added (0, 10, 25, 75, 200, 1000 and 3000 µM) and samples were incubated for 8 minutes at 25°C. Results were analyzed by SDS-PAGE. The figure presenting ATP concentration scale for XPT was graphically modified by removing lines showing protein import after thermolysin post-treatment. 10% represents 1/10 of translation product used for import, p indicates precursor protein, i indicates intermediates whereas m indicates mature form of the protein.

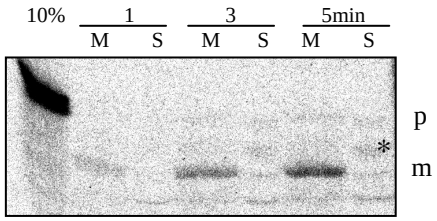
5.5 Chloroplast fractionation into membrane and soluble fractions after import

Considering the hypothesis which describe two protein import pathways into chloroplasts (“stop transfer” and “conservative sorting”) as well as results obtained in the pulse-chase import experiments, proteins chosen for this study were imported into chloroplasts and the organelles were subjected to fractionation into a membrane and a soluble fraction.

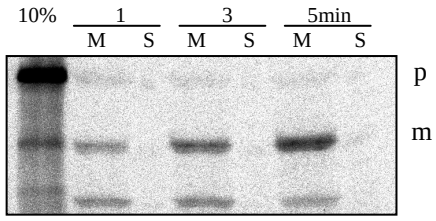
Radioactively labelled precursor proteins were imported into isolated intact chloroplasts for 1, 3 and 5 minutes under standard conditions (section 4.4.1). After re-isolation of intact organelles, samples were treated with thermolysin to deplete precursors bound to the chloroplasts surface. Intact chloroplasts were lyzed in 10 mM HEPES/KOH pH 7.6 for 20 minutes on ice and subsequently membranes were separated from the soluble fraction. The chimeric construct tpS-110N-mSSU (Lübeck *et al.*, 1997) was previously shown to be imported into the inner envelope membrane via a stromal intermediate, therefore it was used as a control for the “conservative sorting” pathway. The mature form of this protein was found both in the membrane and the soluble fraction of chloroplasts.

Among proteins containing a “single” transit peptide, similar results were observed for HP36 and XPT for which some soluble mature forms of the proteins were visible (Fig. 6 A, lanes S). It could suggest that these proteins might use the “conservative sorting” pathway for import. However, during import reaction the intensity of the soluble forms of mature proteins increase or at least remains stable in parallel to the portion in the membrane fraction. Therefore it is rather unlikely that they represent a productive translocation intermediate. Moreover, already after the first minute of the import reaction the vast majority of the mature proteins was observed in the membrane fraction, in comparison to the hardly visible proteins in the soluble fraction. In contrast, the mature form of tpSSU-110N-mSSU was found at the early time of import in majority in the soluble fraction. This indicates that import pathways of the control protein and HP17, HP36 and XPT differ from each other. Therefore it can be concluded that these proteins are probably imported via the “stop-transfer” route.

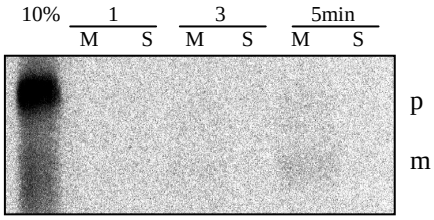
A



HP17



HP28



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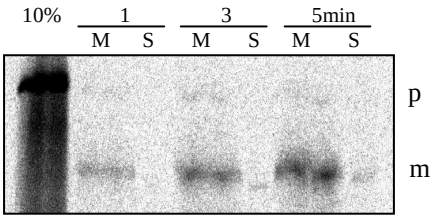
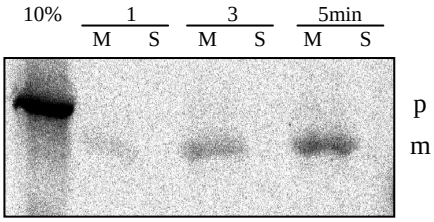
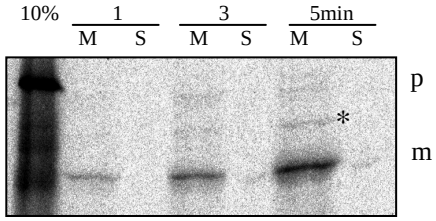


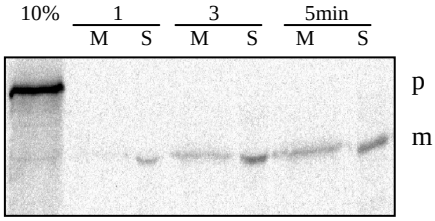
HP36



IEP37

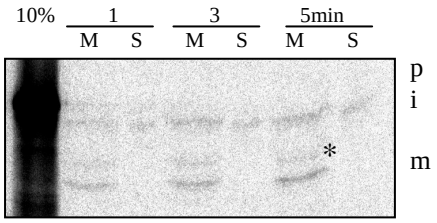


XPT

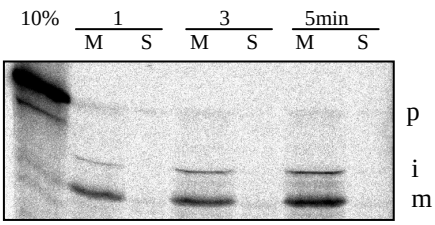


tpSSU-110N-mSSU

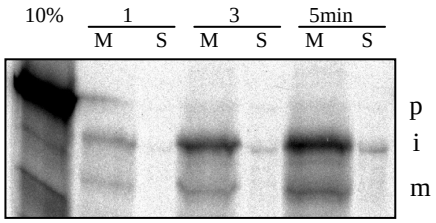
B



HP29b



PIC1



PPT

Figure 6. Suborganellar localization of imported proteins containing a "single" (A) or a bipartite (B) transit peptide
Radioactively labelled precursor proteins were imported into isolated intact chloroplasts under standard conditions (section 4.4.1) for 1, 3 and 5 minutes. Chloroplasts were re-isolated, treated with thermolysin (section 4.4.2) and lyzed in 10 mM HEPES/KOH pH 7.6 for 20 minutes on ice. The centrifugation for 10 minutes at 256,000×*g* separated the membrane fraction (M) from the stromal fraction (S). The construct pSSU-110N-mSSU was used as a control protein for the conservative sorting pathway. p indicates precursor protein, i indicates intermediate, m indicates mature protein, asterisk (∗) indicates either bands of unknown origin (HP17, XPT, HP29b) or precursor protein degradation products which arose after thermolysin treatment (HP28). 10% indicates 1/10 of the translation product used for import experiment.

For HP17, at the third minute of import, apart from the soluble mature form an additional band appeared (Fig 6 A, ∗), which was already observed in previous experiments (Fig. 2 A and 5 A). After the first minute of import reaction this additional band was not visible, in contrast to the mature form of the protein, which was recovered in the membrane fraction (Fig. 6 A). Both the chloroplast fractionation and the pulse-chase experiments (section 5.3) indicate that HP17 does not possess an import intermediate form. For other proteins classified to the first group (Fig. 6 A) a soluble intermediate could not be observed. Already after the first minute of reaction all of the imported mature proteins were detected exclusively in the membrane fractions. For HP28 a shorter fragment than the mature form of the protein was observed in the membrane fraction (Fig. 6 A, HP28, ∗). It might represent peptides arrested within the translocon and truncated after thermolysin digestion. As the amount of mature HP28 increases, the amount of proteolytic fragments decreases from a first to a fifth minute of import. In the case of HP34 the mature protein was only slightly visible after five minutes of import (Fig. 6 A, HP34, lane 5 min, M). Because of the low import efficiency, interpretation of this result was not possible.

The fractionation experiment was especially interesting for the group of proteins that contain a bipartite transit peptide. If the proteins are imported into the inner envelope membrane of chloroplasts according to the "conservative sorting" pathway the soluble stromal intermediates should be observed. Some of HP29b and PPT intermediates were present in the soluble fraction (Fig. 6 B, lanes S) however most probably they do not represent a productive translocation intermediate because they become more intense with increasing import time in parallel to the portion in the membrane fraction. In the group of preproteins containing a bipartite transit peptide only for PIC1 the soluble form was not observed. The intermediate form of PIC1 was present in the membrane fraction of chloroplasts already after the first minute of the import reaction, simultaneously with the mature form of the protein (Fig. 6 B, lanes M).

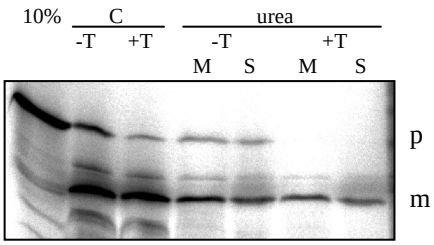
5.6 Protein extraction with 6M urea

According to data presented in proteomics studies (Ferro *et al*., 2002; 2003; Froehlich *et al*., 2003 and Roland *et al*., 2003) and by Eicks *et al*. (2002) preproteins chosen for this study are intrinsic membrane proteins. To confirm the membrane integration inserted proteins were extracted with urea after import.

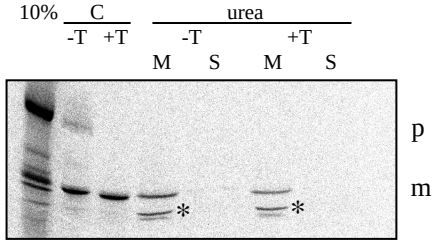
After import (see section 4.4.1) and thermolysin post-treatment (see section 4.4.2) chloroplasts were lysed in 10 mM HEPES/KOH pH 7.6 and divided into the stromal and the membrane fractions. The membrane fraction was treated with 6 M urea for 10 minutes at room temperature. The proteins incorporated into the membranes (Fig. 7 A and B, lanes M) were pelleted whereas proteins extractable with 6 M urea were recovered in the soluble fraction (Fig. 7 A and B, lanes S). The mature forms of HP28, HP34, HP36, IEP37, PIC1, HP29b and PPT (Fig. 7 A and B) could not be extracted from the membrane fraction by treatment with 6 M urea, which suggests their successful integration into the membrane. This fact was confirmed by thermolysin digestion; only precursor proteins were not protected from the protease treatment whereas the mature proteins remained intact. Interestingly, also the intermediate forms of PIC1, HP29b and PPT remained in the membrane fraction after urea treatment. It implied that the processing to both the intermediate and the mature forms of these precursors occurred either when the proteins were arrested within the translocon or after their integration to the envelope membrane. This would be consistent with the low energy requirement (section 5.4), which was optimal for import but was probably not sufficient to drive complete translocation of precursor proteins into stroma. The results obtained after import and urea extraction of HP17 (Fig. 7 A) suggested that the protein might be only partially integrated or loosely bound into the membrane what enabled its extraction by urea. Around 50% of imported HP17 was found in the soluble fraction and this soluble form of the protein was protected from thermolysin digestion. A little amount of XPT was also extracted from the membrane by 6 M urea.

After urea treatment of HP28 and HP29b an additional band appears (Fig. 7 A and B, ∗). Origin of these bands remains unexplained however it might be possible that they represent fragments generated by a urea-induced disruption of the proteins.

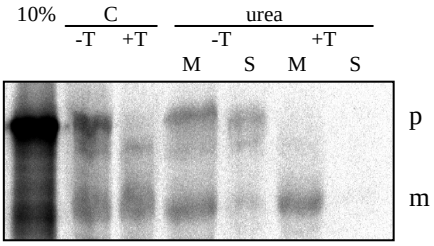
A



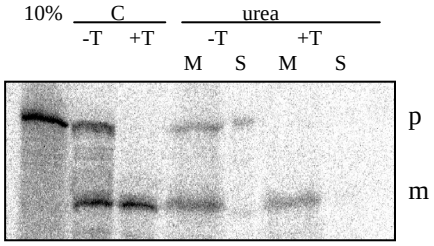
HP17



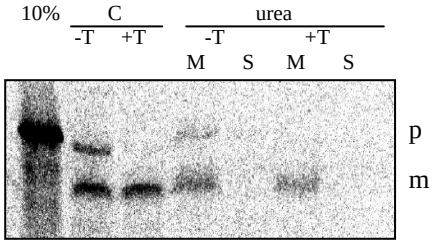
HP28



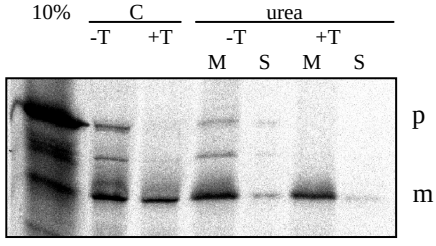
HP34



HP36

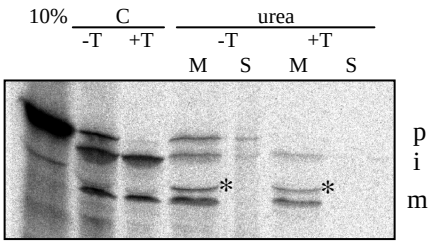


IEP37

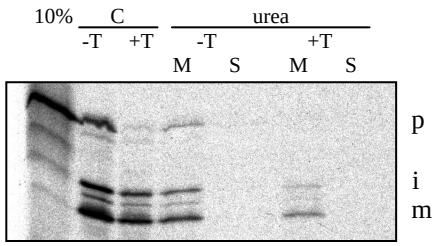


XPT

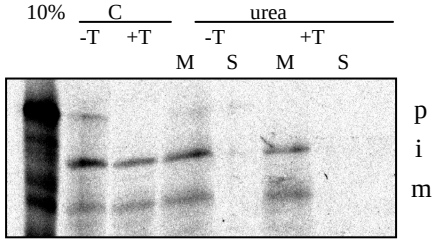
B



HP29b



PIC1



PPT

Figure 7. Protein extraction from the chloroplast membrane fraction by 6 M urea
Radioactively labelled precursor proteins were imported into isolated intact pea chloroplasts corresponding to 40 μg chlorophyll according to standard protocol (see section 4.4.1) and subsequently samples were not treated (-T) or treated (+T) with thermolysin (see section 4.4.2). Chloroplasts were lysed in 10 mM HEPES/KOH, pH 7.6 and divided into membrane and stromal fractions. Proteins from the membrane fraction were extracted by 6 M urea for 10 minutes at room temperature. For the soluble fractions only 50% of the samples were loaded Results were analyzed by SDS-PAGE **A.** Import and urea extraction of proteins containing a "single" transit peptide. **B.** Import and urea extraction of proteins containing a bipartite transit peptide. 10% indicates 1/10 of the translation product used for import experiment, C indicates control samples in which precursor proteins were imported into intact chloroplasts but not treated with urea. Urea indicates samples treated with urea. p indicates precursor protein, i- intermediate form and m- mature form of the protein. The asterisk indicated a novel band appearing upon urea treatment. The lanes on the figure presenting HP29b were not directly side by side on the gel, therefore were graphically modified.

5.7 Competition for import with mOE33 and pOE33

To analyse if the 10 preproteins used in this study share an import pathway with OE33 (33kDa subunit of the oxygen-evolving complex), competition experiments were performed. Radioactively labelled precursor proteins were incubated with chloroplasts corresponding to 15 μg chlorophyll in the presence of 5 μM overexpressed precursor OE33 (pOE33) or mature OE33 (mOE33) protein in import buffer (see section 4.4.1). The import reactions were carried out at 25°C and lasted 10 minutes for all proteins used in this study and 5 minutes for pSSU.

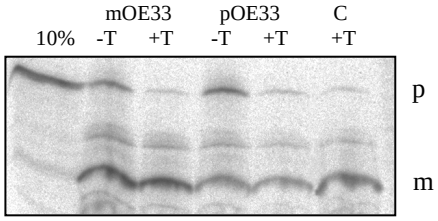
pOE33 is well known to be imported into chloroplasts via the general import pathway, namely the Toc and Tic machineries. If pOE33 uses import components identical with import components used by inner envelope membrane localized preproteins a reduction in the import yield should be observed. mOE33 can not engage the translocation machinery because it lacks the presequence; therefore it was used as a negative control. The results obtained in the competition experiments show that in all cases 5 μM pOE33 inhibited import into chloroplasts, although not to the same extent (Fig. 8 A and B, lanes pOE33). pOE33 exerted the strongest effect on import of PIC1, which was reduced to less than 10% (Fig. 8 B and C). For HP34, HP29b, and PPT (Fig 8 A, B and C) around 20 to 30 % of precursor proteins were imported in the presence of competitor which is similar to results observed for the control protein, pSSU (Fig. 8 A and C). The weakest inhibitory effect on import was observed for HP17 and HP28; more than 60% of radioactively labeled precursors were still imported (Fig. 8 A and C). The control experiment with mOE33

showed that it had no inhibitory effect on the import of any of the precursor proteins tested, on the contrary, HP17, HP28, HP36, IEP37 and XPT in the presence of mOE33 were imported with higher efficiency (Fig. 8 A and B, lanes mOE33). This effect has been observed repeatedly but can not be explained yet. The preproteins examined can be again divided into two groups: the first one containing all precursor proteins with a “single” transit peptide except from HP34 and pSSU, and the second one containing HP34, pSSU and preproteins with bipartite transit peptide. The precursor proteins from the second group behave more like the control protein that suggests similarities in their import pathways. Precursor proteins from the first group seem to deviate from the general import pathway at a certain point, *e.g.* after passing through the outer envelope membrane. Therefore the import inhibition by pOE33 might be less efficient.

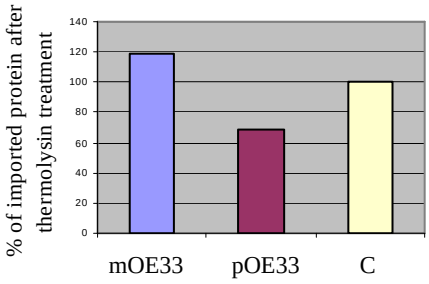
In spite of differences in competition efficiency between preproteins examined, it can be concluded that they seem to use components of the general import pathway at least at the initial import phase.

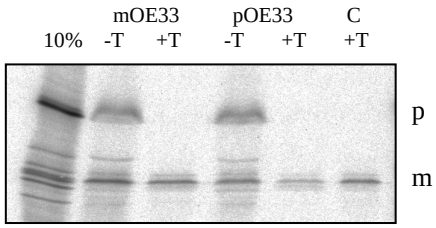
To get a better comparison of results obtained after protein import either in the presence of mOE33 or pOE33, or imported without any competitor, the results were quantified and presented graphically (graphs next to the each figure and Fig. 8 C).

A

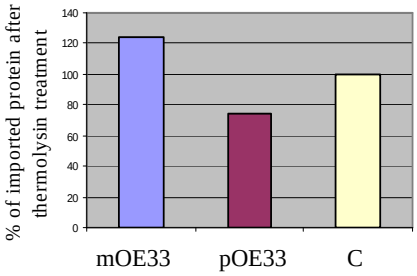


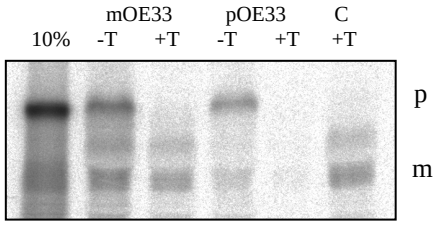
HP17





HP28





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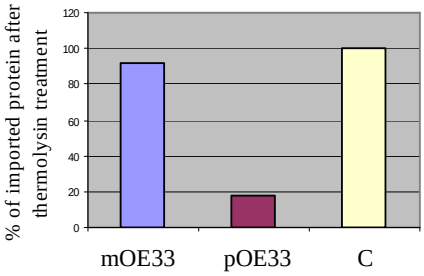

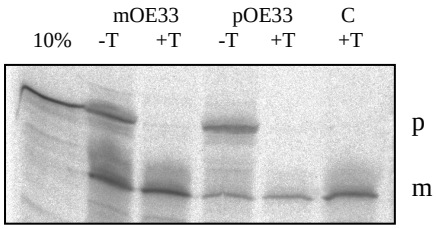

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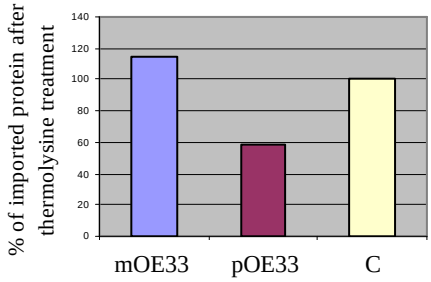
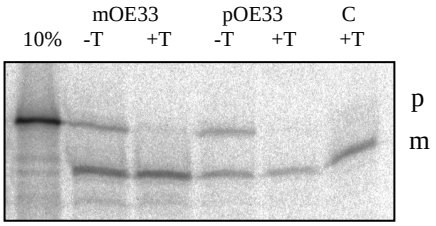
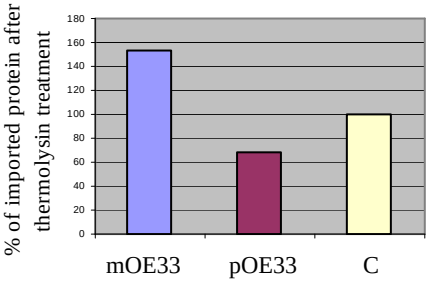
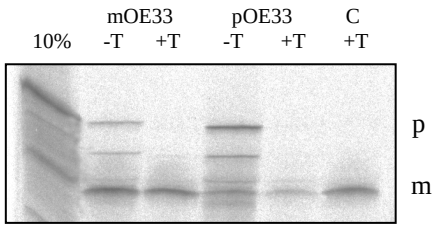




IEP37





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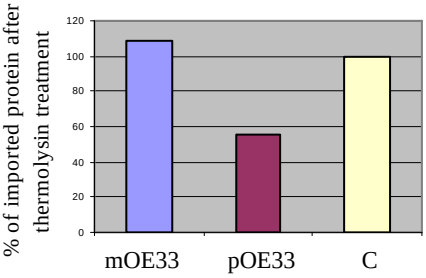
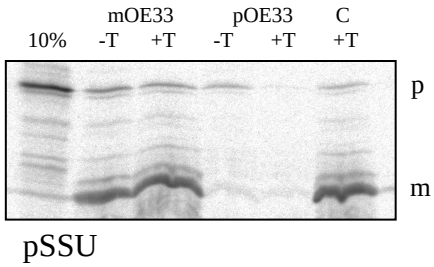
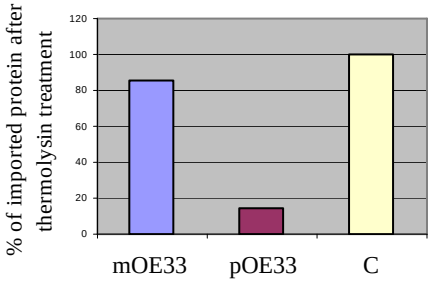
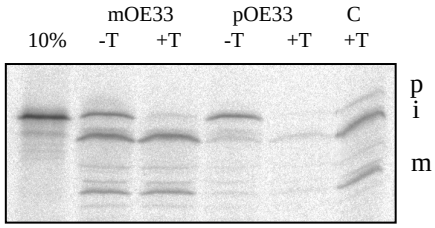




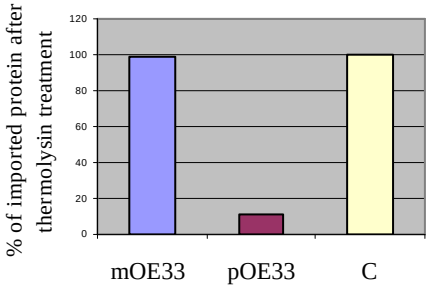
pSSU

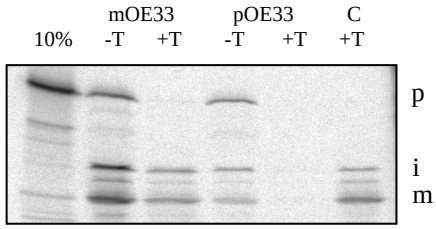


B

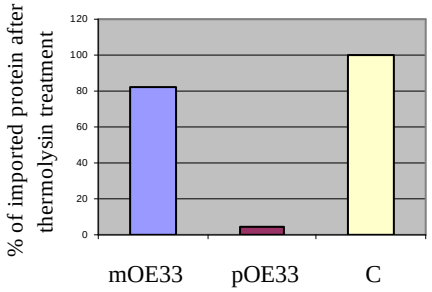


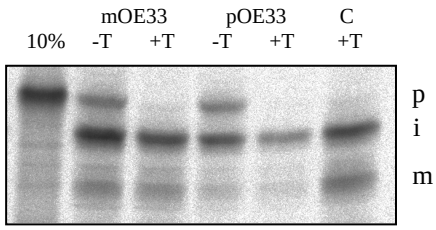
HP29b



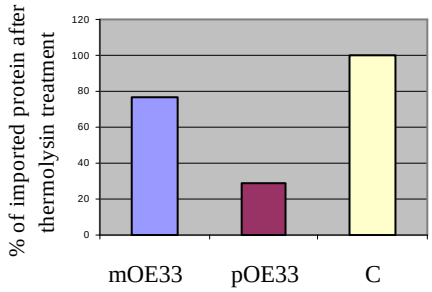


PIC1





PPT



C

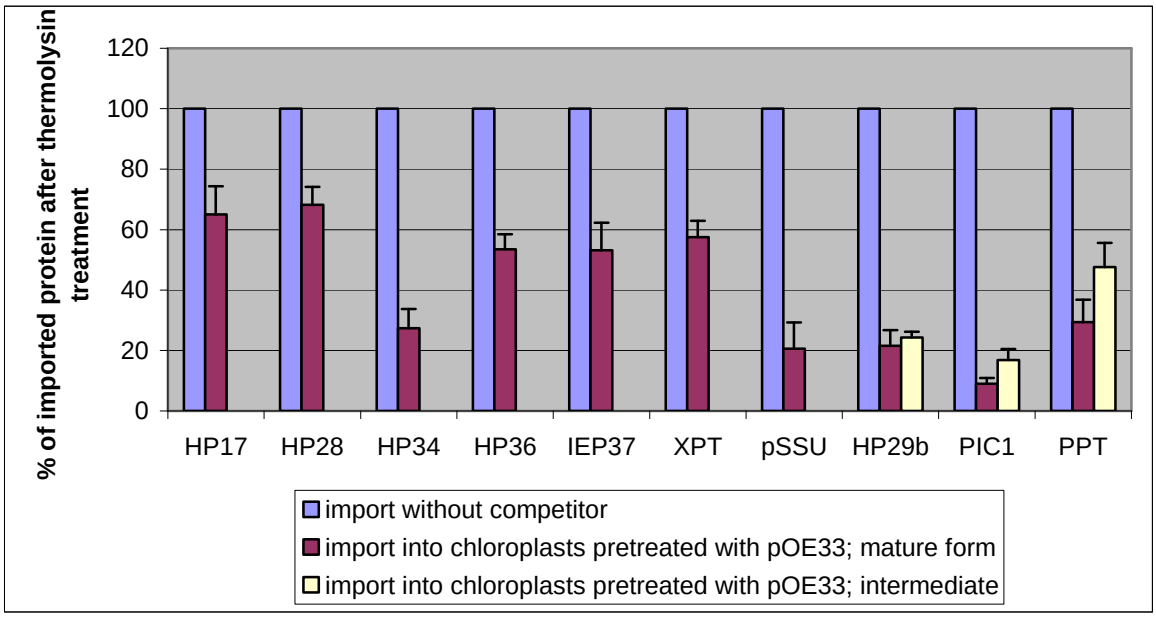


Figure 8. Inner envelope membrane protein used in this approach compete with pOE33 for import into intact pea chloroplasts

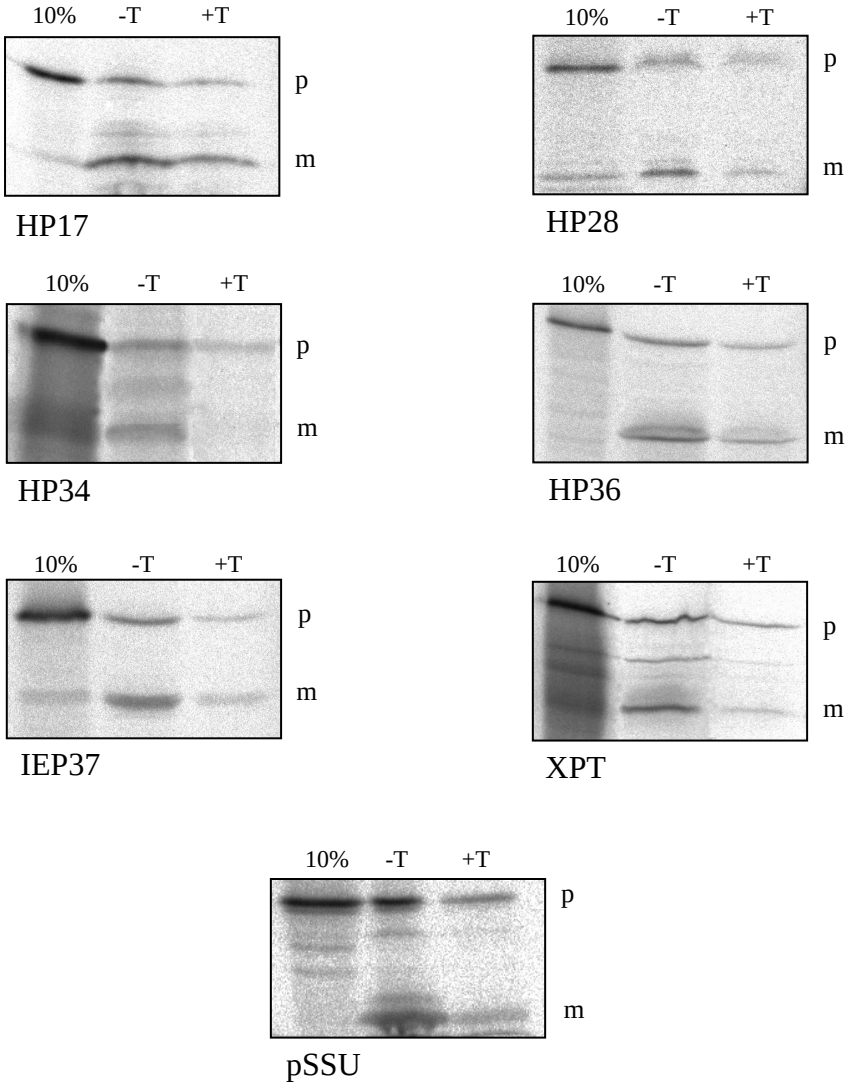
In vitro translated, radioactively labelled precursor proteins were incubated with chloroplasts corresponding to 15 μg chlorophyll under standard conditions in the presence or absence of 5 μM overexpressed competitor, pOE33. Samples containing precursor proteins were incubated for 10 minutes and pSSU for 5 minutes at 25°C. The control reactions were performed with 5 μM overexpressed mature form of OE33 (mOE33) as well as without competitor (C). Samples were treated (+T) or not treated (-T) with thermolysin to remove precursors loosely bound to the chloroplasts surface. Results were analyzed by SDS-PAGE. **A.** Import of precursor proteins containing a "single" transit peptide. **B.** Import of precursor proteins containing a bipartite transit peptide. Results obtained in each experiment were quanified and presented graphically. The control (C) was set to 100%. **C.** The comparison of pOE33 competition effect on import of precursor proteins used in this approach estimated on the basis of three (HP28, HP34, HP36, IEP37, XPT), four (HP29b and PIC1), five (HP17 and PPT) or six (pSSU) independently performed competition experiments. 10% indicates 1/10 of the translation product used for import experiment, p indicates precursor protein, i- intermediate form and m- mature form of the protein.

5.8 Chloroplast pre-treatment with thermolysin

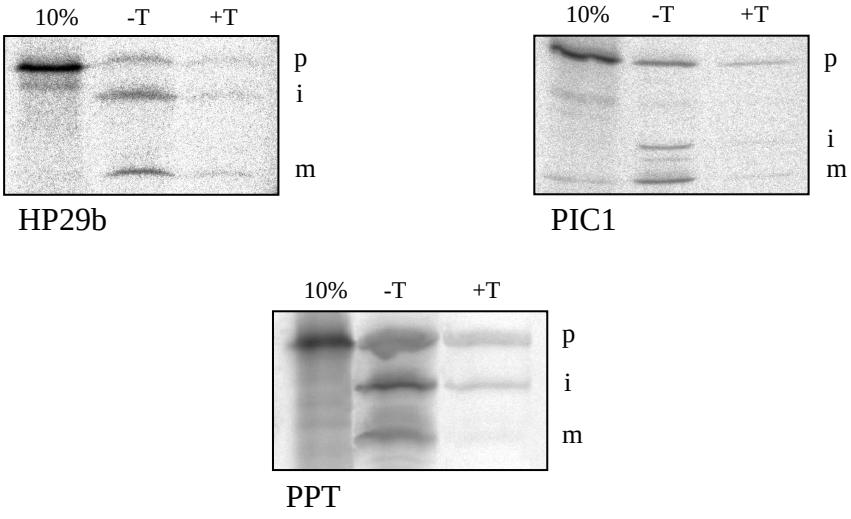
The previous approach, competition for import by overexpressed pOE33 suggested that the inner envelope membrane proteins use components of the general import pathway for import. To test this hypothesis further as well as to better characterize the import pathway at the level of the outer envelope of chloroplasts, intact organelles were treated with 1 mg thermolysin per 1 mg chlorophyll for 30 minutes on ice in the dark. Thermolysin pre-treatment proteolytically removes the soluble parts of receptor proteins (Toc34, Toc64 and Toc159), abolishing their function. Under these conditions import across the two envelope membranes via the general import pathway is greatly reduced (Cline *et al*., 1984; Cline *et al*., 1985).

After thermolysin pre-treatment chloroplasts were re-isolated through a Percoll cushion in the presence of 5 mM EDTA and used for import experiments. In a control sample chloroplasts were not treated with thermolysin. Each reaction contained radioactively labelled translation product in standard import buffer (section 4.4.1) and chloroplasts corresponding to 15 µg chlorophyll. Import lasted 5 minutes for pSSU (control) and for all other proteins used in this study 7 minutes at 25°C. Import of pSSU is known to be dependent on thermolysin sensitive receptors on the chloroplast surface. Therefore thermolysin pre-treatment of intact chloroplasts greatly reduced import of the control protein into the organelle (Fig. 9 A). Similar to pSSU, all other proteins used in this study showed reduced binding to the chloroplasts surface as well as reduced import rates (Fig. 9 A and B, compare lanes –T to +T). This suggests that all preproteins use protease-sensitive receptor components at the surface of the outer envelope membrane. However, it is clearly visible that import into thermolysin-pretreated chloroplasts of HP17 was not as strongly inhibited as import of all other proteins. It might indicate that it is able to bypass the initial recognition step more easily and start the import process directly at the import pore Toc75, which was shown to contain a preprotein binding site (Hinnah *et al*., 2002). In the case of HP28, HP34 as well as for proteins containing a bipartite transit peptide, PIC1 and PPT, removing of the receptors had a stronger influence on their import (around 60% to 70% inhibition). To better compare import efficiency of all precursor proteins obtained results were quantified and presented graphically (Fig. 9 C).

A


10%
-T
+T
p
m
HP17
HP28
HP34
HP36
IEP37
XPT
pSSU

B


10%
-T
+T
p
i
m
HP29b
PIC1
PPT

C

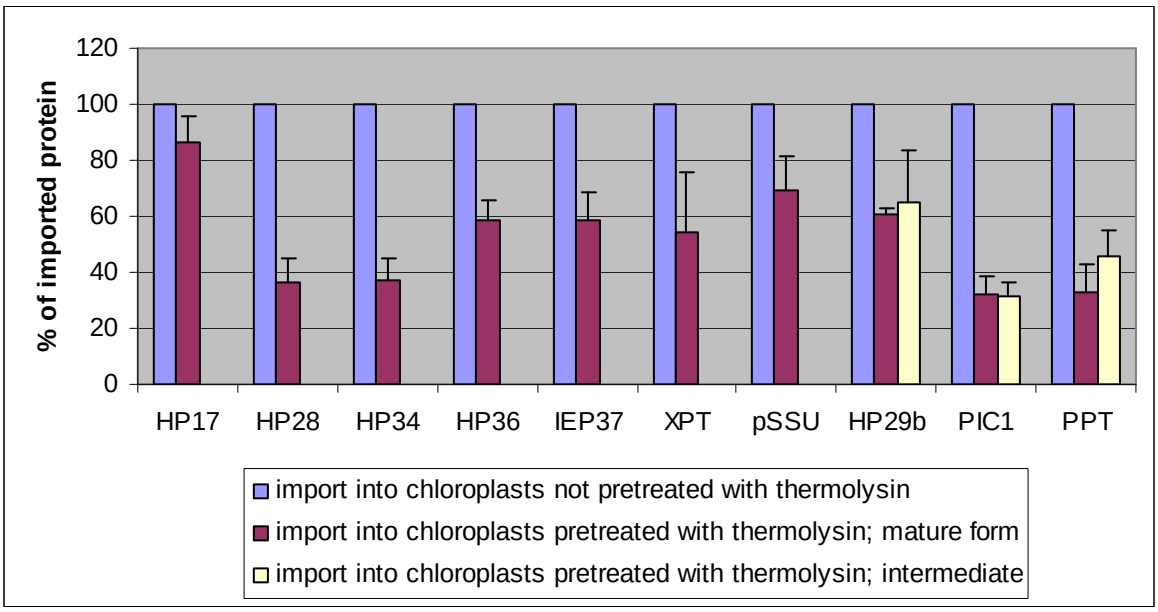


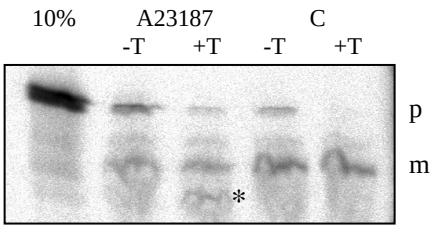
Figure 9. Import into chloroplasts pre-treated with thermolysin
Prior to import chloroplasts corresponding to 1 mg chlorophyll were treated with 1 mg thermolysin for 30 minutes on ice in the dark. Re-isolated and washed chloroplasts corresponding to 15 µg chlorophyll were incubated in import mixture with *in vitro* translated, radioactively labeled precursor proteins for 5 minutes (pSSU) or 7 minutes (HP17, HP28, HP34, HP36, IEP37, XPT, HP29b, PIC1, PPT) at 25°C (+T lanes). Control samples were prepared with chloroplasts not treated with thermolysin (-T lanes). **A.** Import of proteins containing a "single" transit peptide. **B.** Import of proteins containing a bipartite transit peptide. **C.** Quantified and graphically presented results of samples imported into thermolysin pre-treated chloroplasts estimated on the basis of two (HP28, HP34, PIC1), three (HP17, HP36, IEP37, XPT, HP29b), four (PPT) or six (pSSU) independently performed experiments. Samples imported into chloroplasts not pretreated with thermolysin were set to 100%. 10% indicates 1/10 of a translation product used for the import experiment. p indicates precursor protein, i indicates intermediate and m indicates mature form of the protein. The order of lanes on the figures presenting HP34 and PPT was changed graphically.

5.9 Chloroplasts pre-treatment with Ophiobolin A and the ionophore A23187

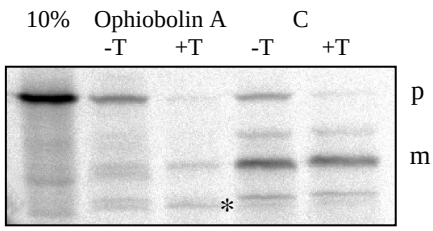
The next step for characterizing import pathways of nine inner envelope membrane proteins concerns the behaviour at the level of the inner envelope membrane. For this purpose two chemical compounds were used: Ophiobolin A and the ionophore A23187. Ophiobolin A specifically inhibits the calcium dependent interaction of calmodulin with its targets by binding to the active site of calmodulin. The ionophore A23187 allows Ca^{2+} ions to cross cell membranes by disrupting transmembrane ion concentration gradients required for proper functioning of the cell. Both Ophiobolin A and A23187 affect the import of pSSU as well as other proteins that possess a cleavable transit peptide and are imported into chloroplasts via the general import pathway. Differently, import of Tic32, which does not possess a cleavable transit peptide was shown to be imported independently of components of the general import machinery, was not inhibited by Ophiobolin A (Chigri *et al*., 2005). Both pSSU and Tic32 were used as the positive and negative control in these experiments, respectively. Because for all proteins the inhibitory effect on their import was stronger if Ophiobolin A was used, Tic32 was imported only in the presence of this inhibitor (Fig. 10 A).

Prior to import chloroplasts corresponding to 2 mg chlorophyll were treated with 100 µM Ophiobolin A or 100 µM A23187 for 20 minutes at 25°C or on ice, respectively. The import reaction (as described in section 4.4.1) of radioactively labelled HP17, HP28, HP34, HP36, IEP37, XPT, HP29b, PIC1 and PPT lasted 10 minutes and import of pSSU and Tic32 lasted 5 minutes. Half of each sample was treated with thermolysin to verify successful translocation. Control samples were prepared with chloroplasts treated neither with Ophiobolin A nor with A23187. Both chemicals reduced the import rate of almost all proteins used in this study, although A23187 inhibited import not as strongly as Ophiobolin A. Only Tic32 was imported into the organelle at the same range, both in the presence and absence of Ophiobolin A in the import mixture. In the case of HP17, HP28, XPT, HP29b and PIC1 the inhibitory effect of A23187 on import was minimal (Fig. 10 A, B and C, A23187), in opposite to the effect observed after Ophiobolin A treatment (Fig. 10 A, B and C, Ophiobolin A). However, the obtained results suggest that translocation of the nine investigated proteins across the outer and inner envelope membrane occurs similarly to pSSU, via the general import pathway. These results also support the hypothesis that import of proteins that possess a presequence and cross the chloroplast envelope membranes via the Toc and Tic machinery might be calcium/calmodulin regulated.

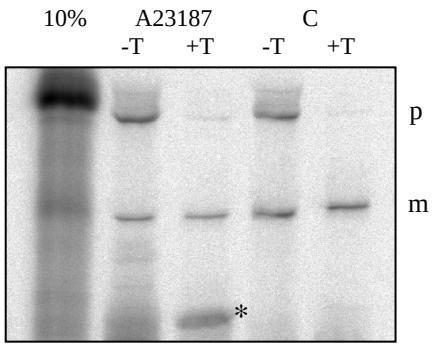
A



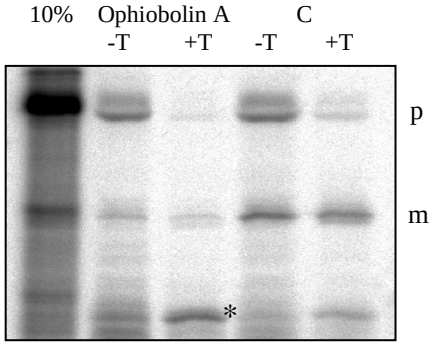
HP17



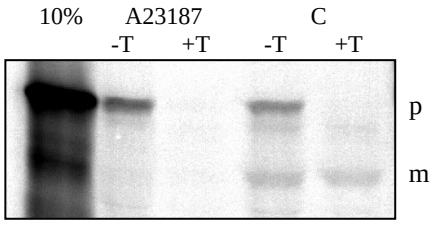
HP17



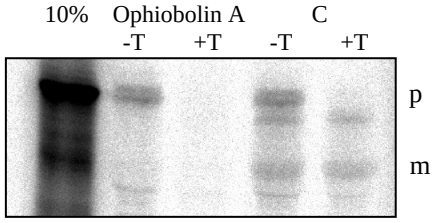
HP28



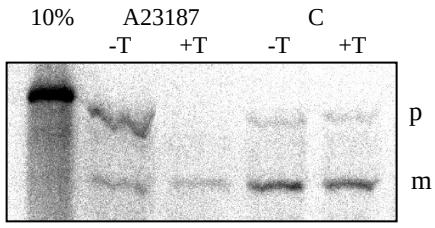
HP28



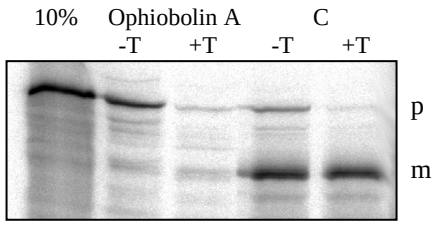
HP34



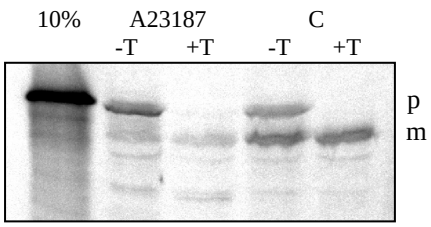
HP34



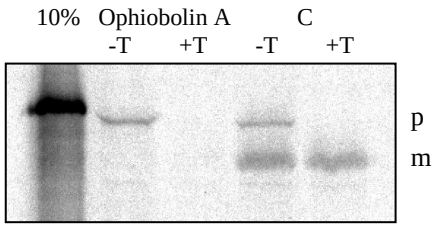
HP36



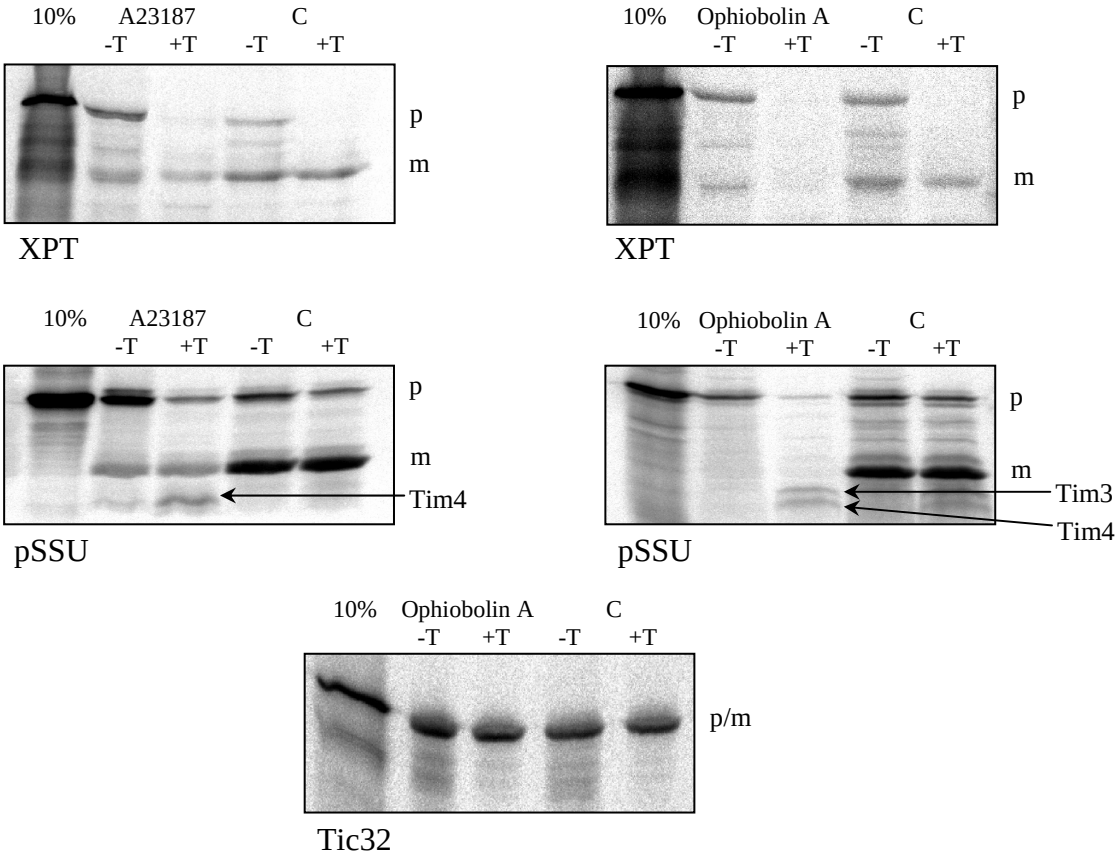
HP36



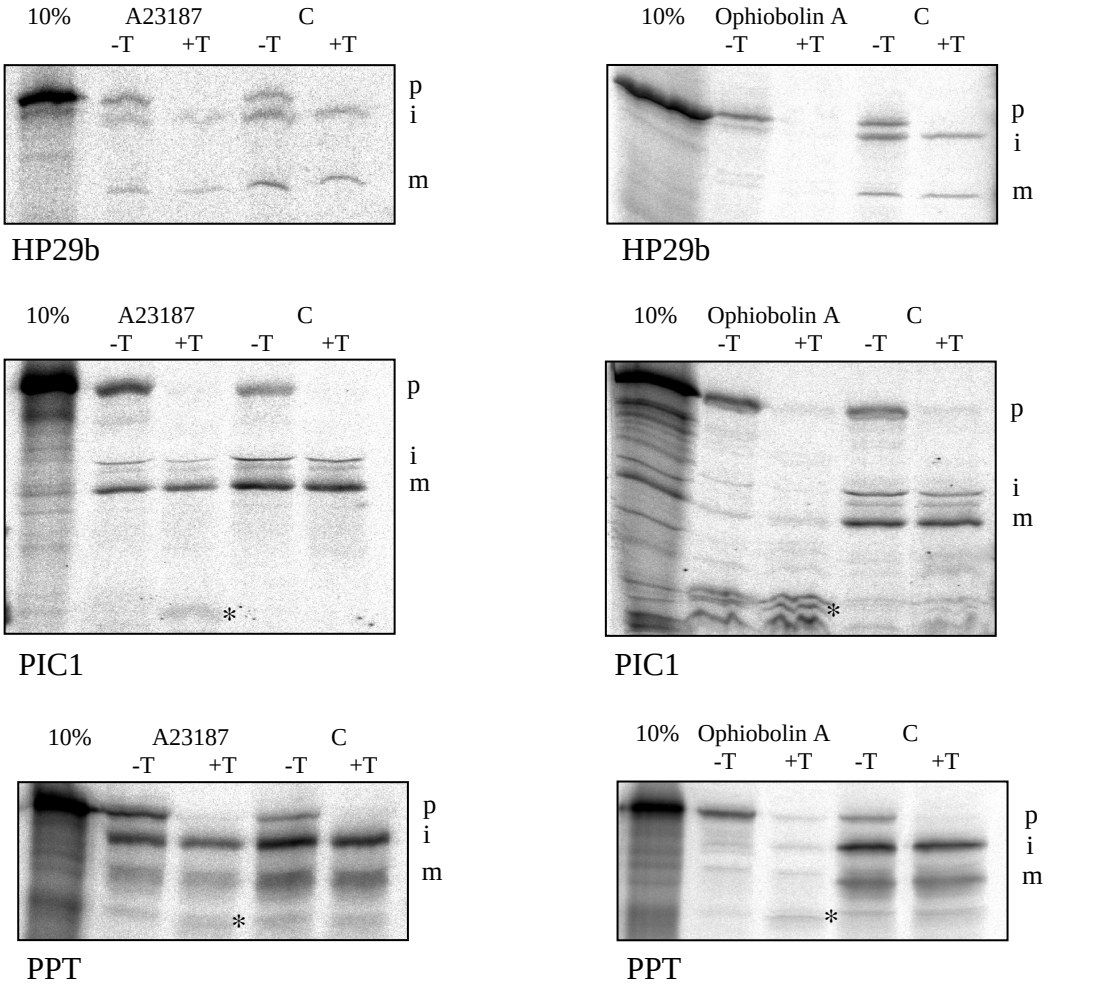
IEP37



IEP37


10%
A23187
C
-T
+T
-T
+T
p
m
XPT
10%
Ophiobolin A
C
-T
+T
-T
+T
p
m
XPT
10%
A23187
C
-T
+T
-T
+T
p
m
Tim4
pSSU
10%
Ophiobolin A
C
-T
+T
-T
+T
p
m
Tim3
Tim4
pSSU
10%
Ophiobolin A
C
-T
+T
-T
+T
p/m
Tic32

B


10%
A23187
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-T
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HP29b
10%
Ophiobolin A
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HP29b
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p
i
m
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PIC1
10%
Ophiobolin A
C
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C

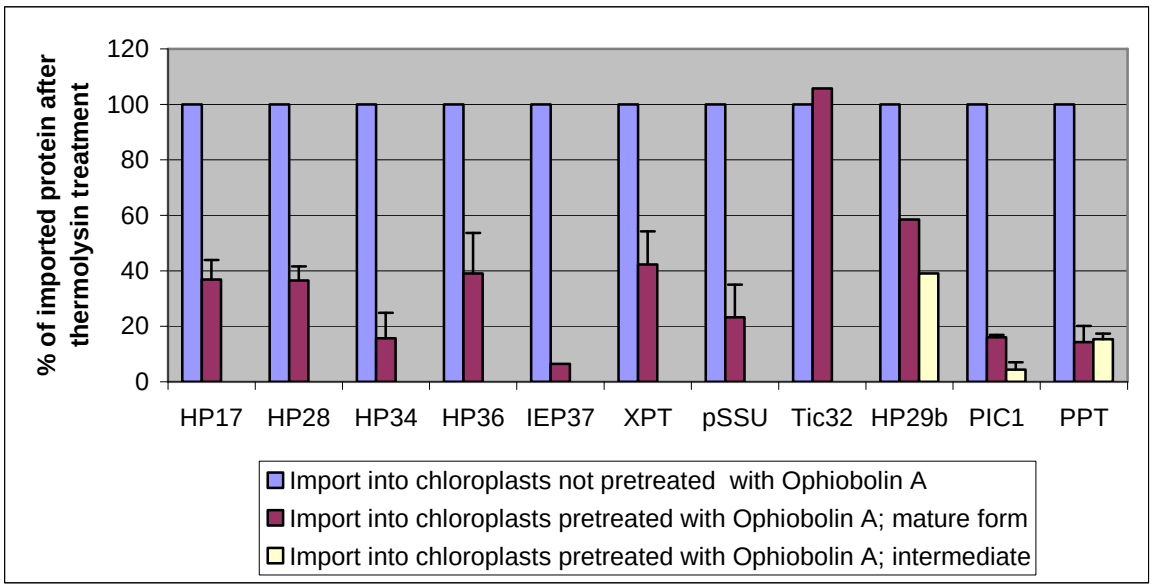

Figure 10. Efect of Ophiobolin A and the ionophore A32187 on import into chloroplasts
Radioactively labelled precursor proteins were incubated with chloroplsts pretreated either with 100 μM Ophiobolin A on ice for 20 minutes or with 100 μM A23187 ionophore at 25°C for 20 minutes. The import reaction was carried out for 10 minutes for HP17, HP28, HP34, HP36, IEP37, XPT, HP29b, PIC1 and PPT, and for 5 minutes for pSSU. In the control samples (C) chloroplasts were treated neither with Ophiobolin A nor with A23187. After import chloroplasts were not digested (-T) or digested (+T) with thermolysin (5 μg of thermolysin per 10 μg chlorophyll) for 20 minutes on ice. Thermolysin digestion was stopped by adding 2.5 mM EDTA, pH 8.0. For HP17, HP28, PIC1 and PPT translocation intermediates were observed after thermolysin treatment (*), similar as for pSSU (Tim 3 and Tim 4). **A.** Import of preproteins with a "single" transit peptide. **B.** Import of preproteins with bipartite transit peptide. **C.** Graphical presentation of the influence of Ophiobolin A pretreatment of chloroplasts on import of all samples used in this experiment, after thermolysin treatment, estimated on the basis of one (IEP37, Tic32, HP29b), two (HP17, HP28, HP34, HP36, XPT, PIC1, PPT) or three (pSSU) independently performed experiments. The control samples treated with thermolysin were set as 100%. 10% indicates 1/10 of a translation product used for the import experiment, p represents precursor protein, i represents intermediate and m-mature form of the protein.

In the case of pSSU import inhibition with Ophiobolin A and A23187 was confirmed by the appearance of the translocation intermediates of about 10 kDa called Tim 3 and Tim 4 (Friedman and Keegstra, 1989; Waegemann and Soll; 1991). Tim 3 and Tim 4 represent proteolytic fragments arrested within the translocon which appear after thermolysin treatment. Similar degradation fragments were observed for HP17, HP28, PIC1 and PPT and were marked by asterisk. It might indicate that those proteins are partially translocated across the outer envelope and at the later stage of import are halted,

presumably at the inner envelope. Differently, HP34, IEP37, XPT and HP29b were completely degraded by thermolysin in the presence of Ophiobolin A in the import mixture.

Because the influence of Ophiobolin A pretreatment of chloroplasts on import was greater than the influence of A23187 pretreatment of chloroplasts, only results obtained in this approach for preproteins imported into chloroplasts pretreated with Ophiobolin A were quantified and presented graphically (Fig. 10, C).

6. Discussion

The knowledge about protein import into chloroplasts is constantly increasing, however, limited studies on protein targeting into the outer and inner envelope membranes have been conducted. Thus, a lot of questions remain unanswered regarding import pathways of membrane proteins. This study broadens the knowledge about protein import into the inner envelope membrane of chloroplasts of α-helical, hydrophobic proteins. We show the complexity of transport process taking into consideration two hypotheses called "conservative sorting" and "stop-transfer".

In the initial import experiments performed in this work nine proteins (HP17, HP28, HP34, HP36, IEP37, XPT, PIC1, PPT and HP29b) from *Arabidopsis thaliana* were successfully imported into isolated pea chloroplasts *in vitro* (Fig. 2 A and B). All of them contain a transit peptide which mediated their transport into the organelle. The envelope membrane localization of all proteins was determined by proteomics studies (Ferro *et al*., 2002; Ferro *et al*., 2003; Froehlich *et al*., 2003 and Roland *et al*., 2003) as well as reported in a research article from Eicks *et al*. (2002), in the case of XPT.

For many inner envelope membrane proteins a cleavable transit peptide seems to be necessary for targeting to chloroplast as in the case of triose phosphate/phosphate translocator (TPT; Brink *et al*., 1995), Tic110 (Lübeck, 1997) or Tic40 (Li and Schnell, 2006), although two exceptions have been characterized so far, chloroplast envelope quinine oxydoreductase (ceQORH, Miras *et al*., 2002; 2007) and Tic32 (Nada and Soll, 2004). It is noticeable that not for all proteins used in this study the length of predicted transit peptide matched the size of the cleaved one. For example, the length of PIC1 transit peptide was predicted according to computational analysis to be 15 amino acids (Chloro P prediction, Table 3), whereas on the basis of N-terminal sequencing and immunoblotting it was determined to be 81 amino acids long (Teng *et al*., 2006; Duy *et al*., 2007). Interestingly, import experiments of PIC1 into isolated pea chloroplasts revealed a thermolysin-resistant intermediate form (Fig. 2 B, PIC1). The same pattern was observed by Duy *et al*. (2007) after import into Arabidopsis chloroplasts. Independent import experiments into pea chloroplasts for the same preprotein were performed by Teng *et al*. (2006). After chloroplast fractionation into stroma, outer and inner envelope membrane, and the thylakoid fraction, they also found a band migrating slightly slower than the mature protein in the inner envelope membrane fraction. Teng *et al*. (2006) described this intermediate form as a rarely observed band of unknown origin. Our studies provide

evidence that PIC1 is imported most probably via processing intermediate. Similar to PIC1, two other proteins, HP29b and PPT, seem to be imported via intermediates which were protected inside the chloroplast from thermolysin degradation (Fig. 2 B, HP29b and PPT). The presence of an import intermediate indicates that the protein possesses a bipartite transit peptide. This type of presequence is common for preproteins transported from the cytosol into the thylakoid lumen (Hageman *et al*., 1990; Ko and Cashmore, 1989) but not for outer or inner envelope membrane proteins, with two exceptions: Toc75 (Inoue *et al.*, 2005) and Tic40 (Tripp *et al*., 2007). The existence of proteins containing a cleavable bipartite presequence was described also in mitochondria. The N-terminal part of a bipartite presequence leads precursor proteins to the mitochondrial matrix, whereas the C-terminal part of a presequence constitutes a sorting signal responsible for insertion of the protein into the inner membrane or to the intermembrane space of the organelle (Pfanner and Geissler, 2001).

On the basis of the presence or absence of an import intermediate all precursor proteins used in this study were divided into two groups. The first group consisted of HP17, HP28, HP34, HP36, IEP37 and XPT, whereas the second group consisted of HP29b, PIC1 and PPT, which were processed twice (Fig. 2 A and B).

For the majority of nuclear-encoded chloroplast proteins which contain a transit peptide the processing is carried out by the stromal processing peptidase (SPP). Much less is known about processing of precursor proteins containing a bipartite transit peptide. Inoue *et al*. (2005) characterized import of Toc75 precursor protein and showed that the first part of its transit peptide is cleaved off by SPP whereas the second processing takes place in the intermembrane space and is carried out by type I signal peptidase, Plsp1, which is most likely localised at the inner envelope membrane with the active domain oriented towards the intermembrane space. On the basis of only one example it can not be considered that every protein containing a bipartite transit peptide is processed similarly to Toc75. Moreover, since the involvement of Plsp1 in the second processing of Tic40 (Tripp *et al.*, 2007) was not confirmed, the involvement in processing of other proteases should not be excluded. In the intermembrane space of mitochondria at least a few processing peptidases have been described so far, which mediate cleavage of precursor proteins directed to the intermembrane space. In some cases the double processing of one precursor protein, *e.g*. cytochrome b_2, is carried out not only by one of these intermembrane space peptidases but also, at the *trans* side of the inner membrane, by the mitochondrial processing peptidase (Neupert and Herrmann, 2007).

For most of the investigated precursor proteins processing seems to be carried out by SPP. It was corroborated by stromal processing assays. Proteins that possess a “single” transit peptide were processed directly to their mature forms in the stromal extract (Fig. 3 A).

In the case of proteins that contained a bipartite transit peptide, the first processing that releases the intermediate form is expected to take place in the stroma, whereas the second processing to the mature protein could be carried out in another chloroplast compartment, *e.g*. in the intermembrane space. Interestingly, only HP29b was processed according to this hypothesis. SPP was probably involved only in the processing to the intermediate but not to the mature form of the protein (Fig. 3 B, HP29b). However, taking into account that it is impossible to obtain a stromal extract without any traces of intermembrane space, the involvement of an intermembrane space peptidase in the processing of HP29b seems to be debatable. The isolated stromal extract probably contains the intermembrane space peptidases, which should be able to process the intermediate-HP29b to its mature form. On the other hand, the insignificant amount of the intermembrane space processing peptidases present in the stromal extract in comparison to amount of SPP from the stroma does not seem to have any influence on protein processing. Therefore, even if the processing of HP29b takes place *in vivo* in the intermembrane space, in this *in vitro* experiment could not be observed and requires further investigation. In the stromal processing assay performed for PIC1 both the intermediate and the mature form of the protein were observed. This suggests the involvement of SPP in both processing events (Fig. 3 B, PIC1). The processing of PPT to its intermediate in the stroma was not clear because of the strong background and weak radioactive signal of the intermediate form. The mature form of PPT was clearly visible, which indicates that the second part of the bipartite transit peptide was cleaved off by SPP in the stromal extract (Fig 3 B, PPT). Although the control protein, pSSU, was correctly processed under the applied conditions, for the inner envelope membrane proteins with a bipartite transit peptide the same conditions might not be appropriate. The proteins might be folded in a way that the processing sites were shielded within the protein sequence and therefore were not accessible to the stromal peptidase.

Further characterization of the import pathways into the chloroplast inner envelope membrane and description of similarities and differences in protein behaviour in modified *in vitro* import system required observation of different import parameters like energy requirement and import kinetics. For complete translocation of precursor proteins into the

stroma high ATP concentration (> 100 μM) is required (Olsen *et al*., 1989; Theg *et al*., 1989; Waegemann and Soll, 1991). ATP is hydrolyzed within the organelle (Theg *et al.*, 1989). Similarly to mitochondria, molecular chaperones are thought to be ATP-hydrolyzing components in the chloroplast translocation machinery (Jackson-Constan *et al*., 2001). The energy requirement for all inner envelope membrane proteins can not be generalized. The main reason is the sorting pathway they follow. It can be expected that inner envelope membrane proteins following the "stop-transfer" pathway will need less energy in the form of ATP than those being imported according to "conservative sorting" mechanism. Tic32 which was shown to use the "stop-transfer" pathway for import, prior to its attachment to the inner envelope membrane is proposed to be targeted into the intermembrane space and requires less than 20 μM externally added ATP (Nada and Soll, 2004). Similarly low energy requirement (< 50 μM) was also established for Tic22 targeted to the intermembrane space of chloroplasts (Kouranov *et al*., 1999; Vojta *et al*., 2007).

For some precursor proteins used in this study only 10 μM to 75 μM ATP was sufficient to observe the mature forms of those proteins. Each of preprotein tested required 200 μM externally added ATP to reach the inner envelope membrane at the maximal import rate (Fig. 5 A and B). The results obtained suggest that only some parts of the proteins reach the stroma but the proteins are never released from the inner envelope membrane translocon. The protein fragment protruding to the stroma could be processed by SPP and the mature protein might be pulled back and laterally inserted to the chloroplast inner envelope membrane.

Interesting results concerning the energy requirement for import were obtained for HP29b, PIC1 and PPT. Taking into account the kinetics of these three proteins, it was clearly shown that both processing steps took place not at the same time; the intermediate was observed before the mature protein (Fig. 4 B). In general, the proteins that possess a "single" transit peptide were processed and imported faster than those being processed twice (Fig. 4 A). Especially HP17 and IEP37 both needed only one minute to achieve the maximal import rate.

The fact that we could identify two different classes of precursor proteins, with a "single" or a bipartite transit peptide, suggested the existence of differences in their import pathways. The two mechanisms were considered, the "conservative sorting" and the "stop-transfer" however, this study provide evidences that all inner envelope membrane precursor proteins examined follow most probably the "stop-transfer" pathway. They do

not seem to have a soluble stromal intermediate (Fig. 6 A and B) as it was shown for other inner envelope membrane proteins, Tic 40 (Tripp *et al*., 2007) and Tic 110 (Lübeck et al., 1997; Vojta *et al*., 2007).

The hypothesis that the inner envelope proteins tested follow the "stop-transfer" route seems to be especially probable for IEP37. Brink *et al*. (1995) suggested the "stop-transfer" import pathway for this protein. In this study IEP37 was not present in the soluble fraction of the chloroplasts at any time of import reaction, which suggests its direct integration into the membrane. Interestingly, in mitochondria proteins which contain one transmembrane domain, as in the case of IEP37, can be arrested at the level of the TIM23 complex and be laterally integrated into the inner membrane (Glaser *et al*., 1990; Miller and Cumsky, 1993; Gärtner *et al*., 1995). This fact and the similarity of chloroplast and mitochondrial import machineries would support the finding that IEP37 is imported into the chloroplasts inner envelope membrane according to the "stop-transfer" mechanism.

The membrane integration of all proteins was observed already at the initial stage of import in the approach in which precursors were imported for short time periods and subsequently chloroplasts were separated into membrane and soluble fraction. Obtained results were confirmed by urea treatment performed after complete insertion of proteins into the chloroplast envelope membrane (Fig. 7 A and B). The only exceptions were HP17, distributed almost equally between the membrane and the soluble fraction, and XPT. They could be extracted by urea, but XPT to a lesser extend. Interestingly, both the intermediate and the mature forms of HP29b, PIC1 and PPT were not extractable by 6 M urea. According to these results as well as import kinetics of HP29b, PIC1 and PPT it can be speculated that these preproteins are inserted into the inner envelope membrane in two steps. In the first step the intermediate is formed and inserted into the membrane in a urea resistant fashion. In the second step the mature form is generated and most probably folded and assembled into its native structure. The hypothesis that inner envelope membrane proteins used in this study are processed while being arrested in the translocon or after their integration into the membrane seems to be very probable.

In order to characterize import of inner envelope membrane proteins in the initial phase the competition experiments with overexpressed pOE33 as a competitor were performed. This protein is known to use the Toc and Tic machineries for import (Row and Gray, 2001). Taking into account that all preproteins tested contain a typical chloroplast cleavable transit peptide it was supposed that all of them engage at least parts of the Toc translocon. 5 μM pOE33 were sufficient to decrease import efficiency of all precursor

proteins used in this experiment, although not to the same extent (Fig 8 A, B and C). According to import efficiency proteins could be again divided into two groups. The first group consists of proteins which import efficiency in the presence of competitor was reduced to about 60% of the maximal import rate, like HP17, HP28, HP36, IEP37 and XPT (Fig. 8 A and C). To the second group proteins containing a bipartite transit peptide (HP29b, PIC1 and PPT) were classified as well as HP34 and a control protein, pSSU. pSSU is known to be imported into the stroma via the general import pathway and therefore competed with pOE33 for import. In the presence of competitor, import of HP29b and PPT was reduced to around 20 to 30% and in the case of PIC1 even to less than 10% of the maximal import rate (Fig. 8 C). The competition of proteins classified to the first group seems to be a matter of debate because of the big differences in import efficiency between proteins from both groups. It is possible that the proteins without an import intermediate deviate from the general import pathway at some point and do not compete for import with pOE33 any more. This could be an explanation why their import was only slightly inhibited in the presence of the competitor. Another reason might be a different affinity of precursor proteins for receptors at the outer envelope membrane, Toc34 (Sveshnikova *et al*., 2000; Becker *et al*., 2004a) Toc64 (Sohrt and Soll, 2000) and Toc159 (Schleiff *et al*., 2003b; Becker *et al*., 2004a) which are involved in the initial import phase of precursor proteins.

To investigate if the import of nine inner envelope membrane proteins in general depends on the receptor proteins, intact chloroplasts were treated with the protease thermolysin prior to import. Thermolysin removes the cytosolically exposed domains of the outer envelope receptors diminishing import efficiency of proteins crossing the outer envelope membrane via the Toc complex (Waegemann and Soll, 1995). Both binding and import of all proteins used in this experiment were reduced, although, similarly to the results observed in the competition experiment there was a big difference in their import efficiency (Fig. 9 A, B and C). The weakest import inhibition was observed for HP17; more than 80% of the maximal import rate of this protein was reached that correlates with results observed in competition experiment. The extremely high import efficiency of HP17 could be explained by the fact that this protein was imported faster than other proteins imported simultaneously (compare the data in pulse-chase experiment, Fig. 4 A). For HP17 the time frame used for analysis might have been out of the linear range of import. Another reason might be the existence of a preprotein binding site at the import channel Toc75 (Hinnah *et al*., 2002). The proteins which were efficiently imported into thermolysin pre-

treated chloroplasts might enter the Toc75 channel directly, without initial recognition by the Toc receptors. On the other hand, import into protease pre-treated chloroplasts of HP28, HP34, PIC1 and PPT was diminished to around 30 to 40%. These results rather exclude the direct insertion of these proteins into the channel.

Taking together the results obtained in the competition and thermolysin pre-treatment experiments it can be concluded that despite of quantitative differences in import efficiency of precursor proteins, most probably all of them enter the chloroplast via the Toc machinery. Moreover, it seems that at the early stage of the import pathway a "single" or a bipartite transit peptide has no influence on the protein behaviour. This assumption is not surprising if we compare the chloroplast import pathways to the mitochondrial import pathways. In mitochondria, in which the import machinery is characterized in more detail than the import machinery of chloroplasts, proteins directed into the inner mitochondrial membrane are transported across the outer membrane via the same channel of the Tom complex, Tom40, although they contain very divergent transit peptides or none at all. Import pathways of mitochondrial proteins are differentiated at the level of the inner mitochondrial membrane where two proteinaceous complexes were characterized, Tim22 and Tim23 (Neupert and Herrmann, 2007). In chloroplasts the Tic complex is proposed to be the main import translocon at the inner envelope membrane.

Recently Chigri *et al*. (2005; 2006) proposed import regulation being mediated by calmodulin and calcium at the level of the Tic complex. Precursor proteins containing a cleavable transit peptide, like pSSU and pOE33, were not imported into the chloroplasts pre-treated with Ophiobolin A, a specific calmodulin inhibitor, as well as with the calcium ionophore A23187. On the other hand, import of proteins that do not contain a cleavable transit peptide and do not use the general import pathway for transport, like Toc34 or Tic32, was not inhibited under these conditions.

The comparison of import behaviour of proteins used by Chigri *et al*. (2005) with ten inner envelope membrane proteins selected for this study allowed us to assess if their import pathways might lead through the general import pathway at the level of the inner envelope membrane. Isolated intact chloroplasts were treated with Ophiobolin A as well as with the ionophore A23187 prior to import. Both compounds had an inhibitory effect on import of all tested precursor proteins, stronger if Ophiobolin A was used for inhibition (Fig. 10 A, B and C). It was previously shown that pSSU imported for a short time with low concentration of ATP (100 μM) at 4°C is arrested in the translocation machinery. After thermolysin treatment proteolytic degradation products called Tim 3 and Tim 4

(degradation intermediate of pSSU) were observed (Friedman and Keegstra, 1989; Waegemann and Soll, 1991). The same effect was observed not only for pSSU but also for HP17, HP28, PIC1 and PPT after incubation of isolated intact chloroplasts with Ophiobolin A or the ionophore A23187 prior to import (Fig. 10 A and B, Tim 3 and Tim 4 in the case of pSSU, asterisk in the case of other proteins). It seems to be clear that all proteins used in this study and especially HP17, HP28, PIC1 and PPT were imported through the Toc complex and arrested at the later import stage, at the level of the inner envelope membrane. It suggests the involvement of the Tic machinery in the import process of all proteins used in this work.

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Ehrenwörtliche Versicherung

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

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Erklärung

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

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