1. Introduction

The cyanobacteria can be considered as a primitive chloroplast ancestor. Indeed the widely accepted theory of endosymbiosis states that a free living cyanobacterium was engulfed by a non-photosynthetic eukaryote. This endosymbiosis provided the cyanobacterium with a stable environment with greater chances of survival. The eukaryote on the other hand obtained the benefit of photosynthesis, which allows the formation of carbohydrates from atmospheric carbon dioxide. There is an inherent dilemma when an organism becomes the symbiont of another cell. Both of them carry their own genomes and thus the control of expression must be transferred to the host or at least be highly regulated and coordinated. An eukaryotic organism displays a higher degree of regulation at the transcriptional level and taking this into account it is not a surprise that the majority of the genes in the ancient cyanobacterial chloroplast ancestor were transferred to the host nucleus. It is estimated that about 95 % of the chloroplast genes have been transferred to the eukaryotic nucleolus.

However, the chloroplast has to maintain all of its functional capabilities in addition to novel ones. This means that the proteins needed for e.g. photosynthesis and lipid metabolism has to be transported back to the chloroplast from the cytosol were they have been translated from nucleus derived mRNA. This may sound relatively obvious and simple but it means that all proteins destined for the chloroplast must have some kind of address tag which directs the proteins synthesized in the cytosol to the chloroplast to avoid accumulation in another part of the cell. Indeed most chloroplast proteins have an N-terminal cleavable transit peptide (TP) that acts like a signal directing proteins to the correct location. The protein containing this TP interacts with special machinery at the chloroplast surface. This machinery actually consists of two parts, one at the outer envelope membrane and one at the inner envelope membrane of the chloroplast. They are called TOC and TIC (Translocon at the Outer envelope membrane/Translocon at the Inner envelope membrane of Chloroplasts) and are made up of different proteins some of which are embedded in the membranes and some that are soluble (Figure 1). This TIC and TOC route is generally viewed as the main route for proteins into chloroplasts.

The aim of this thesis is to elucidate which components are needed for proper chloroplast protein import in Arabidopsis focusing on Toc64 and Tic55 as these proteins have been proposed to have functional significance in pea (*Pisum sativum*). These conclusions were based on biochemical studies and import experiments in on other organisms with the majority of experiments conducted in pea. However, *Arabidopsis thaliana* has the advantage of a sequenced genome. Knockout mutants or anti-sense lines can be obtained relatively easy. Furthermore, chloroplasts can readily be isolated from mutant plants and subjected to direct import experiments where one can look for alterations in import as a result of a gene knockout. This makes Arabidopsis a rather attractive candidate for studying chloroplast protein import. In **Paper I** the effect of Toc64 knockouts on chloroplast protein import was studied. An equally thorough investigation was also performed for Tic55 and its closest homolog Protochlorophyllide (Pchlide)-dependent Translocon Component of 52 kDa (PTC52) in **Paper II**. The possible link between auxin and Toc64 was investigated on root morphology level in **Paper III.** Finally, in **Paper IV**, a proteomic experiment was prepared for both Toc64 and Tic55 mutant plants in an

attempt to identify possible interaction partners for these proteins and shed more light on their role in chloroplast protein import events.

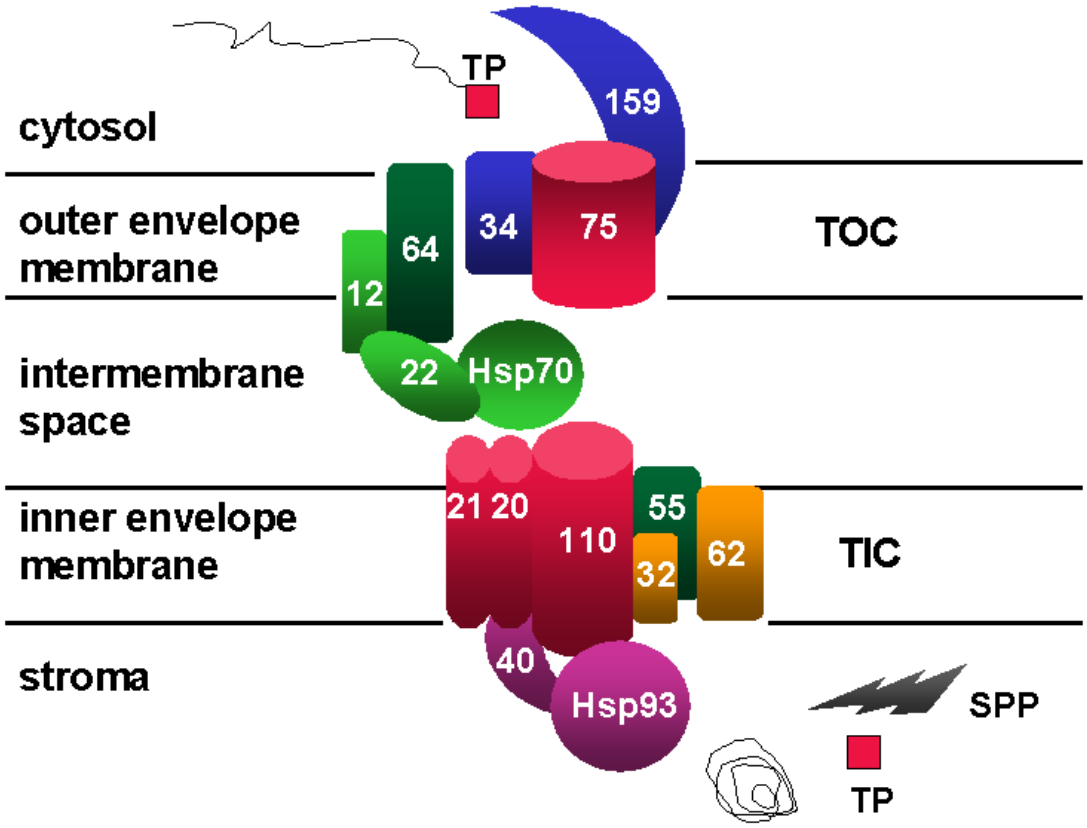

Figure 1. The TOC/TIC translocons of the chloroplast protein import apparatus. The preprotein (black line) is targeted by the help of the transit peptide (TP) to one of the receptors, Toc34 or Toc159 (blue), at the outer envelope membrane. The preprotein is further forwarded to the Toc75 channel (red) before entering the intermembrane space. Here the TOC and TIC is in close contact and by help from proteins in the intermembrane space , Toc12, Tic22 and Hsp70 (light green), the preprotein enters the TIC channel proteins, Tic20, Tic21 and Tic110 (red) at the inner envelope membrane. The preprotein finally reaches the stroma with the help of a motor complex, Tic40 and Hsp93 (purple) and Tic110 (red). In the stroma the TP is cleaved off by a stromal processing peptidase (SPP, grey). The translocation over the inner envelope membrane can also be facilitated by redox-related TIC components, Tic32 and Tic62 (orange). The function of Toc64 and Tic55 (dark green) in unclear. The TOC/TIC components are indicated by their size in kDa.

2. Background

The chloroplast organelle and evolution of the import apparatus

The evolutionary process that changed a cyanobacterial endosymbiont into modern plastids involved not only inheritance but also invention. During the more than one billion years that has passed since the original cyanobacterium became the symbiont of an eukaryotic cell there has of course been a great deal of evolution (Olson 2006). The genome of the chloroplast plastid is in danger of accumulating deleterious mutation due to the lack of sexual recombination (Martin and Herrman 1998). Other than the increased control of gene expression this is probably the other driving force behind the events leading to transfer of genes from the chloroplast to the nucleus. Modern chloroplasts retain many of the biochemical pathways that are plastid specific. The nuclear gene transcripts are translated in the cytosol where the proteins await further processing and transport. The process required for translocation across the two envelope membranes of the chloroplast consists of a large number of protein components. The exact number is currently under debate but we can assume that there are at least 20 components linked to chloroplast protein import in *Arabidopsis thaliana* (Aronsson and Jarvis 2008, Kessler and Schnell 2009, Balsera et al 2009). Because gram-negative bacteria lack a system for polypeptide import, the envelope translocon complex of the general protein import pathway was the most important invention of organelle evolution. It resulted in a pathway to import back into plastids those nuclear-encoded proteins supplemented with a TP. Genome information of cyanobacteria, phylogenetically diverse plastids, and the nuclei of the first red alga, a diatom, and Arabidopsis thaliana allows us to trace back the evolutionary origin of currently known translocon components and to partly deduce their appearance during evolution (Reumann et al 2005). Development of the envelope translocon was initiated by recruitment of a cyanobacterial homolog of the protein-import channel Toc75 (Figure 1), which belongs to a ubiquitous and essential family of Omp85/D15 outer membrane proteins of gram-negative bacteria that mediate biogenesis of beta-barrel proteins. Likewise, three other translocon subunits, Tic20, Tic22, and Tic55 (Figure 1), and several stromal chaperones have been inherited from the ancestral cyanobacterium and modified to take over the novel function of preprotein import (Kalanon and McFadden 2008). Most of the remaining subunits seem to be of eukaryotic origin, recruited from pre-existing nuclear genes. The next subunits that joined the evolving protein import complex likely were Toc34 and Tic110 (Figure 1), as indicated by the presence of homologous genes in the red alga *Cyanidioschyzon merolae*, followed by the stromal processing peptidase, members of the Toc159 receptor family, Toc64, Tic40 (Figure 1), and finally some regulatory redox components, Tic32 and Tic62 (Figure 1), which were probably required to increase specificity and efficiency of preprotein import (Kalanon and McFadden 2008).

Fundamentals of chloroplast protein import

The TP required for the proper localization of chloroplast proteins acts as a flag directing the preproteins exclusively to the correct destination (Figure 1, Smeekens et al 1986).The N-terminal TP is not simply a specific sequence of amino acid residues. It is believed to consist of three separate domains: an uncharged N-terminus, a

central part lacking acidic residues and finally the C-terminal part rich in arginine residues (von Heine et al 1989, Rensink et al 1998). Although these common features have been identified no consensus sequence or structure is known to exist to date. This makes it hard to predict chloroplast localization solely based on sequence analysis (Bruce et al 2000). However, with the general progress in bioinformatics several algorithms are publicly available for chloroplast localization predictions e.g. TargetP, PSORT and ChloroP (Emanuelsson et al 1999, 2000, Nakai and Horton 1999). The importance of the TP in the early stages of preprotein import were characterized and found to be energy dependent. If no energy source is present the binding of the TP to the outer envelope membrane is reversible and actual translocation is not possible (Perry and Keegstra 1994). At ATP concentrations lower than 100 µM and in the presence of GTP the binding is irreversible, however the import is halted at this stage (Young et al 1999) and higher ATP concentration is required for full membrane penetration to occur (Pain and Blobel 1987). At the intermediate stage the preprotein has penetrated the outer envelope membrane and is also interacting with the inner envelope membrane (Wu et al 1994, Ma et al 1996). It is assumed that the high concentration of ATP needed to drive the initial steps of import is attributed to molecular chaperones acting on the translocated protein (Theg et al 1989). It is clear that the TP is crucial at the early stages of protein import and the evolutionary selection for such a system must have been strong. In summary, the TP plays two major roles; one as the address tag for proper subcellular localization and the other as a moderator in the first interaction between preprotein and the components of chloroplast protein import translocon.

Events at the outer envelope

In the simplest model the preprotein interacts directly with components of the outer envelope membrane. This is an attractive model and may hold true for a lot of different preproteins in import experiments. However, some evidence exists for the involvement of lipids in the outer envelope membrane (Bruce et al 1998). The rationale for the lipid involvement in the import process is that the lipids would change the conformation of the lipid bilayer enabling a closer contact between components of the import machinery. Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are the most abundant lipid compounds in the chloroplast membrane (Douce and Joyard 1990). The role of MGDG in protein import has been a subject of debate. An MDGD-deficient mutant did not show a reduction in protein import rates (Aronsson et al 2008) supporting an earlier study (Schleiff et al 2003b). In contrast earlier studies noted an influence of MGDG on protein import (Chupin et al 1994, Pilon et al 1995, Bruce 1998, Schleiff et al 2001, Hofmann and Theg 2005c). An important difference in the experiments conducted by Aronsson et al (2008) was the use of intact chloroplasts instead of artificial lipid bilayers. Nevertheless, it is important to note that this mutant did only display a 40% reduction in the MGDG content and that the MGDG null mutant is probably to severely impaired for import experiments (Kobayashi et al 2007). In contrast to MGDG the DGDG lipid was found to interact directly with the TOC complex (Schleiff et al 2003). Furthermore, a DGDG-deficient mutant revealed a significant defect in protein import experiments (Chen and Li 1998). This mutant showed a 90 % reduction in the DGDG content, which may in part explain the more sever effects found in the DGDG mutant as compared to the MGDG mutant (Dörmann et al 1995). Although tempting, it is to early to rule out MGDG as a lipid important for protein import when several *in vitro*

experiments suggests an important role for MGDG (Chupin et al 1994, Pilon et al 1995, Bruce 1998, Schleiff et al 2001, Hofmann & Theg 2005c).

An alternative model explains the earlier stages of import with the formation of a "guidance complex", involving receptors and chaperones that forms a functional unit together with the TP of the preprotein (Qbadou et al 2006). The TP was suggested to interact with 14-3-3 proteins as well as Hsp70 proteins (May and Soll 2000). It was also demonstrated that chemical modification in the form of phosphorylation of the TP was required for the formation of a guidance complex (May and Soll 2000). Proteins needed at high levels under certain conditions may take the guidance complex route since import of the preprotein of the small subunit of Rubisco (pSS) was 3-4 times faster in the presence of the guidance complex (May and Soll 2000). However, the evidence supporting this concept is relatively weak since only a few proteins have been examined. Mutating the phosphorylation sites in the TP, thought to interact with the guidance complex, did not alter translocation capabilities of green fluorescent protein (GFP) labeled pSS (Nakrieko et al 2004). In summary, the importance of this complex is unclear. In addition, one receptor of the outer envelope membrane, Toc159, could also migrate to the cytosol or possibly a cytosolic form of the receptor would interact with the preprotein forming the initial contact and the first step in the import process (Hiltbrunner et al 2001b).

3. Protein incorporation at the envelope

Outer envelope targeting

Many of the analyzed outer envelope proteins have intrinsic, non-cleavable targeting information. This information is contained within a hydrophobic transmembrane span adjacent to a C-terminal positive region. The C-terminal region separates the proteins from those that enter the endomembrane system since proteins destined to the endoplasmic reticulum (ER) also possesses a hydrophobic transmembrane span (Lee et al 2001). In addition, a cytosolic mediator of outer envelope membrane targeting known as ankyrin repeat protein (AKR2A) was identified. AKR2A acts directly on the protein targeting signal and prevent aggregation before subsequent docking at the membrane surface (Bae et al 2008). The AKR2A mutant shows reduced levels of outer envelope membrane proteins, which is not unexpected if a key role for AKR2A is implicit. More interesting was perhaps that also other chloroplast proteins were affected by the defective outer envelope membrane system. This highlights the importance of a functional outer envelope membrane for normal chloroplast biogenesis. It is also speculated that the AKR2A protein is part of the guidance complex since binding to the 14-3-3 proteins was detected (Bae et al 2008). In addition, a component of the core chloroplast protein import machinery, Toc75, was also indicated for involvement in outer envelope membrane insertion although more research on the exact function of this component for this kind of action is required (Tu et al 2004).

Inner envelope and intermembrane compartment targeting

Research on the targeting of proteins to the intermembrane space is currently quit sparse. Information is limited to no more than two proteins; MGD1 and Tic22 (Figure

1), both are located at the inner envelope membrane surface facing the intermembrane space (Kouranov et al 1999, Vojta et al 2007). Since they both carry a TP they were assumed to take the normal TIC/TOC machinery route. Later it was demonstrated that MGD1 most likely uses the TIC/TOC machinery whereas the information is less clear for the targeting of Tic22. In this scenario the hydrophobic transmembrane domains induce the lateral exit of MGD1 from the TIC machinery followed by membrane integration (Li and Schnell 2006). Thus, these two proteins represent two different models of inner envelope membrane targeting. There is also a difference in the removal of the TP between the two proteins were Tic22 does not seem to be cleaved by the normal stromal processing peptidase (SPP, Figure 1) but instead by an unknown protease probably located in the intermembrane space. The "post import" route of Tic22 suggests that integration into the membrane occurs from the stromal side after import through the TIC complex (Tripp et al 2007).

Proteins targeted to the inner envelope membrane also exist that do not carry a clevable TP. An example is the translocon component Tic32 (Figure 1) where ten N-terminal amino acids contain the targeting signal. Cross-linking pulled out the Tic22 protein, which may aid Tic32 in the process of inner envelope membrane assembly (Nada and Soll 2004). In addition Tic32 does not seem to be dependent on the standard import route through the TOC complex and is probably capable of insertion without chaperones as the insertion occurs even at very low ATP concentrations. Proof also exist that the targeting information does not necessarily lie in the N-terminal part of the protein for proteins targeted to the inner envelope membrane. The correct localization of the Quinone Oxidoreductase Homologue (ceQORH) is dependent on approximately 40 residues in the central part of the protein. Just like Tic32 ceQORH do not utilize the normal TOC mediated entry into the inner envelope membrane. However, higher energy levels are required which may indicate the involvement of chaperons at some point (Miras et al 2007).

4. The TOC complex

Receptors

Two GTPases were first identified in pea as being involved in preprotein recognition and binding (Hirsch et al 1994, Kessler et al 1994, Seedorf et al 1995). These components were later referred to as Toc34 and Toc159 (Figure 1, Table 1). In pea only the Toc34 (psToc34) isoform has been identified but two homologs exist in Arabidopsis, atToc33 and atToc34, which are both very similar to psToc34 revealing approximately 60 % identity (Jarvis et al 1998). Several Toc34 isoforms also exist in maize (*Zea mays*), spinach (*Spinacia oleracea*) and the moss *Physcomitrella patens* (Reumann et al 2005).

Binding of GTP is necessary for Toc34 to receive incoming proteins and carry out its receptor function (Kouranov and Schnell 1997). In one scenario the structure of Toc34 changes upon GTP hydrolysis and the preprotein is then released for further import. Alternatively, Toc159 is the primary receptor and Toc34 binds to the Toc159-preprotein complex. Crystallization of psToc34 in the GDP bound state pointed towards the fact that the receptor can dimerize. In this model the GTPase in one receptor acts as a GTPase activator for the opposite receptor (Sun et al 2002, Bos et al 2007). It is also speculation that Toc159 and Toc34 can heterodimerize and that this process is vital for proper assembly of the TOC complex (Wallas et al 2003).

Table 1. The proposed functions and domains/motifs of the different TOC/TIC components.

Component	Main Arabidopsis isoform	AGI Acc no.	Proposed function(s) (domains/motifs)	First referred to in the literature
Toc12	atToc12	At1g80920	Co-chaperone (Dna J)	Becker et al (2004)
Toc34	atToc33	At1g02280	Preprotein receptor (GTPase)	Kessler et al (1994)
Toc64	atToc64-III	At3g17970	Receptor and unknown (TPR and amidase)	Sohrt and Soll (2000)
Toc75	atToc75-III	At3g46740	Import channel (ß-barrel)	Waegemann and Soll (1991)
Toc159	atToc159	At4g02510	Preprotein receptor and import motor (GTPase)	Hirsch et al (1994)
Tic20	atTic20-I	At1g04940	Import channel	Kouranov et al (1998)
Tic21	atTic21	At2g15290	Import channel and permease	Sun et al (2001)
Tic22	atTic22-IV	At4g33350	TOC-TIC interaction	Kouranov et al (1998)
Tic32	atTic32-IVa	At4g23430	Redox/calcium sensing (short chain dehydrogenase)	Hörmann et al (2004)
Tic40	atTic40	At5g16620	Co-chaperone (TPR and Sti1)	Stahl et al (1999)
Tic55	atTic55-II	At2g24820	Redox sensing and unknown (mononuclear iron site and Rieske iron-sulfur centre)	Caliebe et al (1997)
Tic62	atTic62	At3g18890	Redox sensing (NAD(H) dehydrogenase)	Küchler et al (2002)
Tic110	atTic110	At1g06950	Import channel and chaperone recruitment (TP- and Tic40-binding sites)	Schnell et al (1994)
Hsp93	atHsp93-V	At5g50920	Import motor (ClpC/Hsp100 and Walker ATPase)	Shanklin et al (1995)

The binding properties of Toc34 have been proposed to be controlled by phosphorylation as the unphosphorylated protein is unable to bind GTP (Sveshinkova et al 2000). The actual site of phosphorylation was confirmed to be a serine residue at position 113 in psToc34 and at position 181 in atToc33 (Jelic et al 2002, 2003). However, substituting the serine at position 181 in atToc33 did not alter the activity of the protein (Aronsson et al 2006). Thus, it is interesting that the regulation by phosphorylation in the two Arabidopsis orthologs occurs at different positions and possibly by different mechanisms. Phosphorylation of psToc34 was ascribed to a certain kinase with unknown identity (Fulgosi and Soll 2002). Although most groups generally accept the proposed receptor function of Toc34 there is still speculation as to which receptor, Toc159 or Toc34, is the primary one. Whether or not Toc34 is the primary receptor it is interesting to note that the two isoforms in Arabidopsis appear to have specific preferences for different types of proteins (Kubis et al 2003). A Toc33 mutant showed lower levels of photosynthetic proteins whereas housekeeping proteins remained at a stable level (Kubis et al 2003). The Toc34 and

Toc33 mutants have growth phenotypes; the Toc33 mutant is pale and the Toc34 mutant has shorter roots but normal photosynthetic tissues (Jarvis et al 1998, Constan et al 2004b). In addition, a Toc34/Toc33 double mutant was found to be embryo lethal (Constan et al 2004b).

The Toc159 component, first identified in pea, consists of four members in Arabidopsis; atToc159, atToc132, at Toc120 and atToc90 (Perry and Keegstra 1994, Hiltbrunner et al 2001b). The atToc159 and psToc159 are the most similar components and share a 48 % identity on amino acid level making them the true functional orthologs (Bauer et al 2000). Toc159 has three distinct domains: an N-terminal acidic domain (Kessler et al 1994), a GTPase domain (Table 1) and a hydrophilic M domain thought to act as a membrane anchor (Hiltbrunner et al 2001a). Interestingly, the membrane anchor does not consist of typical transmembrane helices (Bauer et al 2000). Toc159 is believed to be the primary receptor for incoming preproteins in the “targeting model”.

Cross-linking studies usually pulls out a lot of Toc159-preprotein aggregates (Kouranov and Schnell 1997) and an antibody raised against Toc159 blocks the formation of early import intermediates (Hirsch et al 1994). Furthermore, Toc159 was also detected in the cytosol (Hiltbrunner et al 2001b) and soluble Toc159 was found to interact exclusively with the TP of preproteins (Smith et al 2004). In this model there is a cycle flow of Toc159 between the cytosol and the membrane surface. Toc159 is proposed to play a role similar to the signal recognition particle (SRP) of ER protein import and this would imply that Toc34 has a role similar to that of the SRP receptor. The actual insertion of Toc159 into the outer envelope membrane was proposed to be mediated by Toc34 in a GTP regulated fashion (Wallas et al 2003) where the heterodimerization of Toc159 and Toc34 would induce GTP hydrolysis. However, the role of this dimerization in relation to GTP hydrolysis is unclear (Sun et al 2002, Yeh et al 2007). In part the occurrence of cytosolic Toc159, a prerequisite in this model, could be attributed to disrupted membranes that contaminated the samples in the experimental procedure (Becker et al 2004a).

In the opposing “motor model” Toc34 is the primary receptor and Toc159 is constantly membrane anchored eliminating the necessity of a cytosolic Toc159 (Figure 1). The protein feeding action of Toc159 towards Toc75 is driven by cycles of GTP hydrolysis (Becker et al 2004a). Toc159 and Toc75 was sufficient for transport into liposomes (Schleiff et al 2003) and upon isolation of the TOC complex Toc34 and Toc75 was found in equal amounts and was found to be approximately three times more abundant than Toc159 (Kikuchi et al 2006). Using a competitive TP fragment, binding of preproteins to Toc34 could be blocked but inhibition of Toc159 did not affect the binding of incoming preproteins (Becker et al 2004a). The GTP bound Toc34 receptor is believed to bind the preprotein and bring it into the proximity of Toc159 were hydrolysis of GTP partly releases the preprotein towards Toc159 which then subsequently hydrolyses GTP causing the protein to enter the Toc75 channel (Schleiff et al 2003). The hydrolysis of GTP by Toc34 causes this component to dissociate from the TOC complex making it ready to act as the primary receptor again. Just as for Toc33/Toc34 the Toc159 proteins and its homologs are believed to act on different targets where Toc159 and Toc33 are part of a specific import machinery for photosynthetic proteins, and Toc120, Toc132 together with Toc34 are needed for import of housekeeping proteins (Bauer et al 2000, Smith et al 2004). However, cross-talk most likely occurs between the pathways.

Channels of the outer envelope membrane

Early cross-linking experiments on preproteins revealed that the Toc75 protein is in close proximity of the import apparatus and is the most likely channel candidate at the outer envelope membrane (Figure 1, Perry and Keegstra 1994). It was one of the first identified TOC components in pea (Waegemann and Soll 1991), and is one of the most abundant proteins in the outer envelope membrane (Cline et al 1981). Import can be inhibited by the addition of a Toc75 antibody (Tranel et al 1995). The channel pore is thought to be about 14 Å in diameter requiring the protein to be unfolded upon entering the channel (Hinnah et al 2002). The membrane topology is made up of 16 lipophilic β-strands forming a typical bacterial barrel structure (Table 1, Schleiff et al 2003). In Arabidopsis there are three homologs to the pea component; atToc75-III, atToc75-IV and atToc75-V. The Toc75-III component is the true homolog of psToc75 with a 73 % sequence similarity and similar expression patterns (Table 1, Baldwin et al 2005). Mutating the *atTOC75-III* gene resulted in embryo lethality proving the importance of this component. The atToc75-IV protein is expressed only at very low levels and is thought to play a minor role in chloroplast protein import (Baldwin et al 2005). Toc75-V carries a bipartite signal that directs entrance into the stroma of the chloroplast followed by a second membrane insertion step similar to the action of the psToc75 component (Inoue and Potter 2004). The size of the mature protein was also found to be around 80 kDa and the name OEP80 was adopted. Homologs of a bacterial beta-barrel protein, Omp85, universally exist in the outer membranes of Gram-negative bacteria, mitochondria and chloroplasts. There are two distinct Omp85 homologs in Arabidopsis; Toc75 and another homolog, OEP80, with unknown function (Hsu et al 2008). It was later shown that OEP80 is essential for viability in Arabidopsis and that the N-terminal region of OEP80 is not essential for the targeting, biogenesis, or functionality of the protein unlike Toc75-III (Patel et al 2008). Unlike Toc75-IV a role for Toc75-V in protein import has been demonstrated and it is believed that this component may help in the translocation of α-helical barrel proteins of the inner envelope membrane and large bulky β-barrel proteins in the outer envelope membrane (Inoue and Potter 2004).

Translocation through the outer envelope membrane

The translocation through the TOC complex was at one point thought to be driven solely by the activities at the TIC complex however more evidence has accumulated that point toward some kind of motor activity in of the outer envelope membrane aiding transfer through the Toc75 channel (Kovacheva et al 2007). In one model the GTPase activity of the Toc159 component causes protein translocation where every cycle of GTP hydrolysis pushes another part of the protein through the membrane. This model is sometimes referred to as the “sewing machine” (Schleiff et al 2003). Although this is an attractive model one cannot neglect the fact that the role of GTP is questionable. Removing the G-domain or using GTP analogs that are not hydrolysable does not affect the import of preproteins (Kessler et al 1994, Chen et al 2000). The involvement of chaperones makes the situation more complex. It is previously known that the formation of the early import complex requires ATP, which may imply the involvement of molecular chaperones in this process (Olsen and Keegstra 1992). Between the TOC and TIC complexes the chaperone Hsp70 may act as a molecular magnet grabbing on to proteins at the TOC complex and making sure that direction occurs in just one direction in a manner similar to mitochondria (Neupert and Brunner 2002). Hsp70 was found in close proximity to the TOC

complex when other components were isolated (Figure 1, Schnell et al 1994). Even more intriguing is that Toc12 was proposed to act as a co-chaperone modulating the action of Hsp70 (Figure 1, Becker et al 2004b). Toc12 is a DnaJ-like protein probably anchored in the outer envelope membrane with the bulk of the protein located in the intermembrane space (Table 1). This part consists of the C-terminal J domain which is thought to function in redox-sensing through the action of a disulphide bond (Becker et al 2004b). Even though the exact role of Toc12 is unclear, it is thought to be a member of the translocase in the intermembrane space.

5. Toc64

Identification

Membrane proteins complexes can be enriched from intact chloroplasts by detergent solubilization. These complexes contain TOC and TIC components as well as preproteins (Schnell et al 1994). A partially purified TOC complex from pea chloroplast envelope membrane was isolated and found to contain TOC subunits Toc34, Toc75, Toc159 and Tic110. In addition, the Hsp70 chaperone and both Rubisco subunits were detected (Waegemann and Soll 1991). Several undefined proteins were also detectable in the isolated active TOC complex and one of these appeared later to be a protein of 64 kDa mass (Figure 1, Sohrt and Soll 2000). Separation was performed on sucrose density gradients with the isolated TOC complex. The cross-linker $CuCl_2$ was found to form the reversible formation of disulfide bridge(s) between Toc64 and the established TOC complex subunits in the purified outer envelope membranes (Sohrt and Soll 2000). This finding led to the adoption of the name Toc64 to emphasize the role of this component in the formation of a functional TOC complex (Sohrt and Soll 2000, **Paper I**). Later, in Arabidopsis, three genes sharing homology with the psToc64 protein were found and given the names atToc64-I, atToc64-III and at Toc64-IV where the roman numerals specify the location on chromosomal level (Jackson-Constan and Keegstra 2001).

Properties

The true ortholog of the psToc64 protein is atToc64-III and so far only this component is known to be chloroplast located (Chew et al 2004). Therefore comparison in a chloroplast protein import aspect should be made between these two components. The Toc64-I protein is smaller than the Toc64-III protein lacking both the tetratricopeptide repeats (TPR) motifs and the transmembrane anchor region, which Toc64-III has (Table 1, **Paper I**). Hence, any functional redundancies between components are not likely to occur (**Paper I, III, IV**). This is further strengthened by observations placing Toc64-I in the cytosol (Chew et al 2004, Pollmann et al 2006). Interestingly the amidase region of Toc64-I appears to be enzymatically active as opposed to Toc64-III and Toc64-V where the active site has been mutated by a single point mutation (Sohrt and Soll 2000, **Paper I**). Indole-3-acetamide (IAM) was found to be hydrolyzed by Toc64-I, which is interesting since this auxin intermediate was recently identified in Arabidopsis (Polllmann et al 2006). This observation relates Toc64-I to auxin biosynthesis and subsequently the name Amidase 1 (AMI1) was adopted (Pollman et al 2006, **Paper III**). Interestingly, the Arabidopsis Toc64-I mutant showed a subtle phenotype in root growth patterns possibly created by alterations in the endogenous auxin levels (**Paper III**). However, if there is an effect it is subtle and

therefore further studies are required and the levels of auxin in Toc64-I mutants should be measured.

The Toc64-V protein is also similar to the psToc64 component but was found to be localized in the mitochondria and was therefore renamed as mitochondrial outer membrane protein 64 kDa (mtOM64) (Chew et al 2004). Just as Toc64-III the Toc64-V protein contains a deactivated amidase region and the TPR motifs, and is of equal size (**Paper I**).

Expression profile of the different atToc64 paralogs

The *Toc64-I* homolog expression is highest in developing rosettes and lowest in the germinating seed (Figure 2A). After rising slightly in the seedlings expression falls again in the young rosettes where expression reaches a value similar to that of the mature siliques (Figure 2A). The expression profile for the chloroplast localized *Toc64-III* protein shows a rather different expression pattern than for *Toc64-I.* The germinating seed has the highest expression level (Figure 2B). After germination the expression drops in the seedlings, young rosettes and the developing rosettes but increases again during bolting to almost the same value as in the germinating seed (Figure 2B). Young and developing flowers display lower expression than under bolting but the lowest expression was found for flowers and siliques (Figure 2B). In the mature siliques expression is some where between that of developed flowers and germinating seeds (Figure 2B). *Toc64-V* has a similar expression pattern to that of *Toc64-III* until approximately the bolting stage. At this stage *Toc64-V* retains its expression value from bolting to mature siliques with a slight dip during flowering (Figure 2C). Just as for *Toc64-III* the expression of *Toc64-V* after germination drops in the seedlings and developed rosettes (Figure 2C). However, there is difference since the expression of *Toc64-V* is as high in the young rosette as it is in the germinating seed (Figure 2C). In summary, the expression profile does not indicate a clearly redundant function between the paralogs **(Paper I)**. In addition, the expression of *Toc64-I* is much more expressed than the other paralogs in 14 day old plants **(Paper I)**.

Toc64 as a receptor

Although the amidase capability of Toc64-I is interesting much more attention has been focused on the TPR domain of psToc64 and later Toc64-III and Toc64-V in Arabidopsis. TPR motifs were first identified in the ssn6 nuclear protein and nuc2+ a protein required for normal mitotic disjunction (Schultz et al 1990, Hirano et al 1990). It has later been found in several proteins with a broad range of functions (DÁndrea and Regan 2003). Of special interest in membrane biology are the translocation receptors of different organelles that also contain this motif. The mitochondrial receptors Tom20, Tom34 and Tom70 all contain the TPR domain (Hauke et al 1996, Yang et al 2002). Other examples include the peroxisomal receptor Pex5 and the translocon component of the ER (Gatto et al 2000, Ponting 2000). Three tandemly arranged TPR motifs form a right handed superhelix. This superhelix structure is capped by a solvation helix at the C-terminal end creating together with the described TPR motifs the 3-TPR domain (Jabet et al 2000). The loops connecting the helices show a higher mobility factor revealed by NMR measurements. Even more interesting is that the amino terminal end and the solvation helix are flexible in terms of positioning and folding (Cheng et al 2006). The 3-TPR domains involved in the recognition of the molecular chaperones Hsp70 and Hsp90 forms a conserved

dicarboxylate clamp that binds to the two carboxylate groups of the C-terminal aspartates in Hsp70/Hsp90 (Scheufler et al 2000). Interestingly the 3-TPR motif of Toc64-III and Toc64-V was described as being of the dicarboxylate clamp type (Qbadou et al 2006). This led to the proposal that pea Toc64 mediates docking of Hsp90-associated pre-proteins (Qbadou et al 2006).

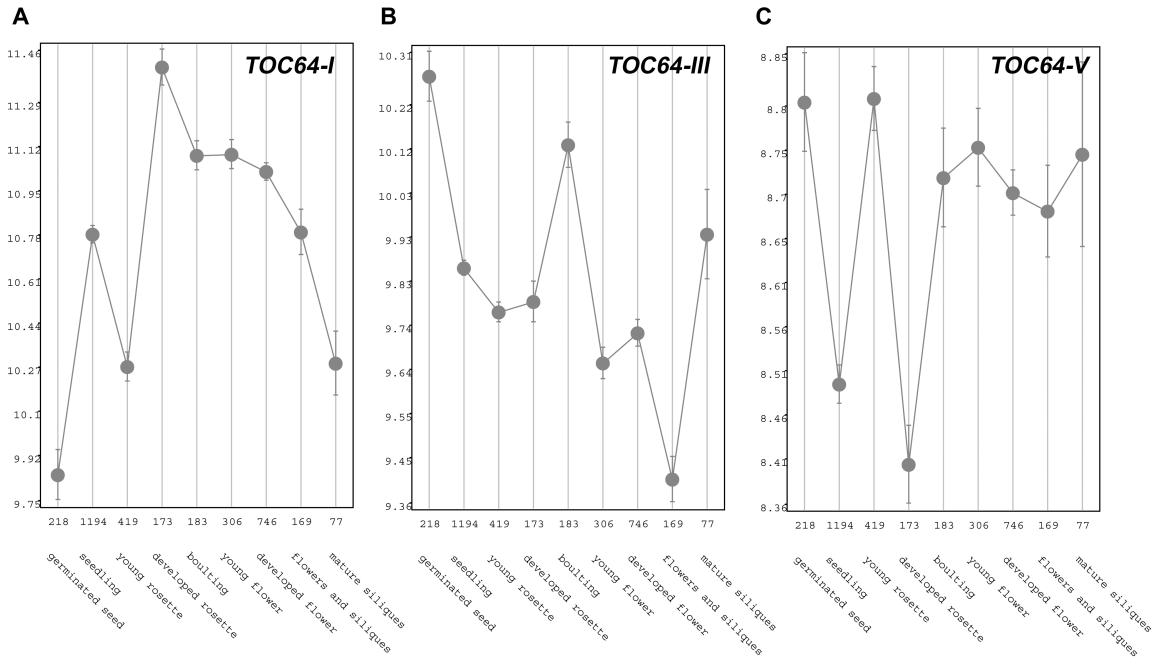

Figure 3. Expression patterns for the *TOC64* genes in Arabidopsis. The expression data for **(A)** *TOC64-I*, **(B)** *TOC64-III* and **(C)** *TOC64-V* was retrieved using Genevestigator V3 (www.genevestigator.ethz.ch/) and prepared with the Meta-Profile Analysis tool. Data from all high-quality ATH1 (22-k) arrays were analyzed and the values shown are means (± SD).

Toc64 was also suggested to be involved in translocation on both sides of the membrane and would in this scenario also act as a regulator of Hsp70 as part of the intermembranes space complex (Becker et al 2004a). These are attractive hypotheses, as they attempt to explain both the remarkable specificity of the import machinery, and the coordination of pre-protein arrival with the activation and assembly of downstream components of the import machinery. However, they are inconsistent with data from several other lines of experimentation. Firstly, preprotein translocation by the TOC complex has been shown to proceed efficiently *in vitro* in the absence of Toc64 (Schleiff et al 2003a). Secondly, and despite alternative proposals, it would appear that the vast majority of the Toc64 protein is oriented towards the cytosol (Lee et al 2004, Hofmann and Theg 2005a), making it an unlikely participant in complex activities in the intermembrane space. The complete loss of a protein with such wide-ranging activities in the import mechanism would be expected to have severe consequences, as numerous other studies have demonstrated that defects in translocon components cause either strong chlorosis, albinism or embryo lethality (Jarvis et al 1998, Bauer et al 2000, Kubis et al 2003, Baldwin et al 2005,

Kovacheva et al 2005). However, no detectable phenotype can be detected when comparing the triple mutant, *toc64-III/V/I* with wild-type plants (Figure 3, **Paper I, III**). Finally, in the proteomic analysis of the Toc64 triple mutant, *toc64-III/V/I*, one would expect to find TOC or TIC components being affected by this mutant. However, only Toc75 was identified as a potential target showing a calculated increase of protein expression at 35 % (**Paper IV**).

As to the role of Toc64-V one can only speculate but since the receptor function of Toc64-III is under debate the same must be considered true for Toc64-V. The hypothesis that Toc64-V would substitute for Tom70 is based on the similarity of there TPR motifs and to a large extent molecular modeling (Mirus et al 2009). No experimental data has been presented on the function of Toc64-V (**Paper I**).

Figure 3. Wild-type, *toc64-III/V/I* and *tic55-II-1* plants. Plants were grown initially for two weeks on MS media according to Aronsson and Jarvis (2002). Then the plants were transferred and grown on perlite-soil at 20℃ for four weeks using a 8 h day/16 h night cycle.

Toc64 in different organisms

Even tough Toc64 homologs have been identified in a number of organisms the experimental data is contained within a few of them (Kalanon and McFadden 2008). Cyanobacteria and the red alga *C. merolae* also appear to lack Toc64 orthologs. Thus Toc64 is likely to be of eukaryotic origin (Reumann et al 2005) probably appearing after the divergence of red algae. Its presence in both prasinophyte and streptophyte genomes indicates that a full-length Toc64 was present before the divergence of higher plants, but a full-length version was either absent or lost from the ancestor of the chlorophyte *Chlamydomonas reinhardtii* (Kalanon and McFadden 2008). However, the prasinophyte orthologs retains the serine residue in the catalytic triad of the amidase region, which distinguishes them from the vascular plant Toc64 proteins (Qbadou et al 2007, **Paper I**).

Since Toc64 was fist identified in pea most biochemical studies started with psToc64 in focus (Sohrt and Soll 2000). Later research turned more towards Arabidopsis (Qbadou et al 2006, 2007, **Paper I, III, IV**). However, there is an inherent disadvantage in using Arabidopsis since the isolation of e.g. envelope membranes is rather difficult and requires a large amount of starting material. This has led some

researchers to keep the pea system when biochemical analysis is performed and reserve the use of Arabidopsis for e.g. genetic or proteomic purposes (**Paper IV**). In 2005 two Toc64-like proteins were identified in the moss *P. patens* (Hofmann and Theg 2005b). A double knockout affecting these two components showed no obvious phenotype and experiments revealed no chloroplast protein import defects. These observations are similar to the results obtained by scrutinizing the Arabidopsis Toc64 triple mutant (Figure 2, **Paper I**). The translocation complexes of *C. reinhardtii* are similar to the model TOC and TIC translocons of Arabidopsis (Kalanon and McFadden 2008). The absence of Toc64, and more specifically its cytosolic TPR domain, suggests that Toc64 is not an absolute requirement for protein import and that *C. reinhardtii* lacks the Hsp90-dependent TP chloroplast-trafficking pathway observed in pea (Qbadou et al 2006). Further weakening this hypothesis is that the oxygen evolving protein of 33 kDa (OE33) was suggested to take the Hsp90/Toc64 route, was imported at normal rates in the Arabidopsis *toc64-III/V/I* mutant (**Paper I**).

Current position of research

Resent studies have focused on bioinformatics and molecular modeling (Mirus et al 2009, **Paper IV**). The TPR motif of Toc64 has been of special interest perhaps since this fold is known to be important in other systems (Mirus et al 2009). However, preprotein translocation does not seem to require a functional Toc64 protein since the core components Toc34, Toc75 and Toc159 was able to import preprotein flawlessly in reconstituted liposomes (Schleiff et al 2003a). Moreover, knocking out a protein with a bipartite function in both the outer envelope membrane and intermembrane space could be expected to result in some kind of phenotype. This does not appear to be the case for Toc64 neither in *P. patens* nor in Arabidopsis (Figure 2, Hofmann and Theg 2005b, Paper I). The function of Toc64 is therefore unclear and if Toc64 functions as a receptor it is probably a member of a non-essential receptor class (Mirus et al 2009, **Paper I**). Interestingly, a proteomic analysis of chloroplasts from the Toc64 triple mutant revealed Toc75-III to be down-regulated by approximately 35 % and the enzyme glyceraldehydes-3-phophate dehydrogenase was found to be up-regulated by approximately 28 % (**Paper IV**). However, what this means for the function of Toc64-III is presently unclear.

It has been suggested that Toc64-V functionally replaces Tom70 in mitochondria. This is base largely on sequence comparisons and on the assumption that Toc64-III behaves as a receptor in chloroplast protein import interacting with Hsp90 (Qbadou et al 2006). There is no experimental data supporting this notion and future experiments may very well identify Toc64-V as a non-essential receptor in mitochondria just as Toc64-III in chloroplasts (**Paper I**). A yeast line with a mutated Tom70 can be used to investigate whether Toc64-V could functionally replace Tom70 or not, and thereby revert the Tom70 mutant phenotype.

The most interesting development in recent years is the discovery that Toc64-I has an active amidase region that can hydrolyze Indole-3-acetamide (IAM), which is a precursor in auxin biosynthesis (Pollmann et al 2006). Moreover the Toc64-I mutant had a root phenotype that can possibly be attributed to altered auxin levels (**Paper III**).

6. Events at the inner envelope

Channel candidates

The identity of the TIC channel is a matter of debate since three different components have been proposed to perform this role. The first Tic110 (Figure 1, Table 1) was identified in pea by its close association with imported preproteins (Schnell et al 1994). Tic110 carries in its N-terminal an approximately 9 kDa membrane anchor consisting of two α-helical membrane spans. The C-terminal part consists of a 98 kDa hydrophilic domain initially assumed to reside in the intermembrane space (Lübeck et al 1996). More recent studies have placed the bulk of the protein in the stroma where it is thought to recruit stromal factors needed for import (Inaba et al 2003). Tic110 was reported to have a pore diameter of 15 Å when reconstituted into liposomes and was also found to have a β-barrel structure. This β-barrel was considered to constitute the actual pore and also displayed cation-selective behavior (Heins et al 2002). However, overexpression of Tic110 in bacteria and plants produced a soluble α-helical conformation of the C-terminus and challenging the previous β-barrel model (Inaba et al 2003). If Tic110 acts as a channel the membrane spanning N-terminal probably mediates the role. This leaves the C-terminal moiety to interact with the incoming preprotein possibly through the interaction of a domain in the C-terminus with the TP of the protein to be imported (Inaba et al 2003). Recently, characterization of Tic110 revealed two regions in the intermembrane space localized to form supercomplexes with the TOC machinery and to receive the TP of preproteins. A large region was also found to reside in the stroma for interaction with molecular chaperones (Balsera et al 2009). The presence of Tic110 in most tissues and it's vide distribution among species confirms this component as one of great importance (Kovacheva et al 2005, Kalanon and McFadden 2008).

The Tic20 (Figure 1) component consists of four transmembrane α-helices and was first identified in pea by its association with preproteins (Kouranov et al 1998). Four paralogs have been identified in Arabidopsis were Tic20-I shares a 63% identity with psTic20 and is generally considered as the true homolog. Tic20 was suggested to play a role in channel formation and shares a weak homology and topology with the mitochondrial Tim23/22/17 translocase components (Table 1, Rassow et al 1999, Reumann et al 1999). Just like Tic110 the Tic20 protein is expressed in most tissues and expression is highest in juvenile plants (Chen et al 2002). Antisense lines of Tic20 had an import reduction defect and were also phenotypically distinguishable by their pale appearance (Chen et al 2002)

Through the aid of forward genetic screening of import deficiency using a selectable marker Tic21 (Figure 1) was identified (Teng et al 2006). The Tic21 protein is similar to Tic20 in terms of size and topology. Expression of Tic21 is similar to those of Toc75 and Tic110 with the exception of germination were Tic21 has a lower expression, suggesting a more active role in later developmental stages (Teng et al 2006). It may also be that Tic20 and Tic21 have a functional overlap. Mutating Tic21 in Arabidopsis results in abolished import at the TIC translocon and an albino phenotype (Teng et al 2006). Interestingly the accumulation of ferritin clusters and differential regulation of genes involved in transport or iron stress led to the conclusion that Tic21 may act as a iron transporter regulating cellular ionic homeostasis (Table 1, Duy et al 2007). Thus, Tic21 is also referred to as Permease In Chloroplasts 1 (PIC 1).

Translocation model of the inner envelope and core components

Compared to the translocation of proteins across the outer envelope membrane the translocation events at the inner envelope membrane are less well characterized (Aronsson and Jarvis 2008, Kessler and Schnell 2009, Balsera et al 2009). Apart from the actual channel proteins the process is also thought to require a motor complex that drives the transport into the stroma and in addition regulatory factors connecting transport to e.g. the redox state of the chloroplast (Aronsson and Jarvis 2008, Kessler and Schnell 2009, Balsera et al 2009).

The complete transfer of preproteins over the inner envelope membrane consumes large quantities of ATP in the stroma (Theg et al 1989). This requirement of ATP can be attributed to stromal chaperones that are part of the motor complex. Components of this motor complex include Tic40, Tic110 and Hsp93 (Figure 1, Aronsson and Jarvis 2008, Kessler and Schnell 2009, Balsera et al 2009). Tic110 carries a TP recognition site close to the TOC channel exit (Table 1, Inaba et al 2003). This interaction is thought to prevent the preprotein from reentering the intermembrane space. A second role of Tic110 is probably to recruit molecular chaperones with its stromal domain (Table 1). The activity of Hsp93 was suggested to be regulated by the co-chaperone Tic40 (Table 1). Genetic interaction data supports the idea that Tic40, Tic110 and Hsp93 works together in close cooperation (Figure 1, Kovacheva et al 2005).

Tic40 (Figure 1) is encoded by a single gene both in pea and in Arabidopsis (Chou et al 2003). It was shown to associated with preproteins at the inner envelope membrane and was first characterized in pea (Ko et al 1995). Tic40 has one transmembrane α-helix at the N-terminus while the C-terminus constitutes a soluble part that protrudes into the stroma (Chou et al 2003). The close association between Tic110 and Tic40 was demonstrated by cross-linking experiments (Stahl et al 1999). The co-chaperone hypothesis first came into focus when it was discovered that Tic40 displays a certain degree of homology with the Hsp70 interacting protein (Hip) and also with the Hsp70/Hsp90 organizing protein (Hop) in a region of the C-terminus referred to as the Sti1 domain (Table 1). Tic40 also possesses a TPR motif upstream of the Sti1 region (Chou et al 2003). Even more interesting was the fact that the Sti1 region could be functionally replaced by the same putative region of the human Hip protein further strengthening the image of Tic40 as a co-chaperone (Table 1, Bédard et al 2007). However, Tic40 is not vital for import since the mutants are pale and only show a partial import defect (Kovacheva et al 2005).

Cross-linking experiments support the involvement of Hsp93 (Figure 1) in protein import at the inner envelope membrane (Akita et al 1997). The interaction is not dependent on preprotein and appears to by destabilized by the presence of ATP (Kouranov et al 1998). In Arabidopsis two genes for Hsp93 have been identified; atHsp93-III and atHsp93-V (Jackson–Constan and Keegstra 2001). The two homologs share a 93 % identity and it is believed that they can act redundantly even though they appear to have a slightly different expression pattern (Kovacheva et al 2005). Indeed, knockout mutants of Hsp93-III showed no phenotypic differences from wild-type plants suggesting redundancy by Hsp93-V (Constan et al 2004a). Hsp93-V mutants were found to have a chlorotic phenotype and reduced import rates (Kovacheva et al 2007). In the same study the double Hsp93-III/Hsp93-V mutant was found to be embryo lethal illustrating the importance of these components.

Action of the motor complex

Although the motor complex theory is an attractive model some issues needs to be addressed such as the interaction between Tic40 and Tic110. At the import intermediate state the TOC and TIC complexes are formed and the preprotein start to associate with the TIC machinery (Inaba et al 2003). This supercomplex already contains the Hsp93 chaperone helping the preprotein to quickly move into the TIC channel. The motor complex of the TIC then comes into operation powered by stromal ATP. The interaction between Tic40 and Tic110 is thought to be mediated by the TPR region of Tic40 and further stimulated by the presence of a preprotein binding at the TP interaction site of Tic110 (Chou et al 2006). The interaction triggers the release of the TP from Tic110 making it accessible for Hsp93. The Sti1 region of Tic40 stimulates the ATPase activity of Hsp93, in a way similar to mitochondrial import (Neupert and Brunner 2002), enabling the chaperone to complete the translocation process true the intermembrane space. Tic40 acts in the last steps of envelope translocation and regulates the action of Tic110 and Hsp93 by controlling the activity of Hsp93 (Chou et al 2006).

Linking the events at the TIC complex to the energetic state

During light conditions energy in the form of ATP is stored and deducing power needed for carbon fixation is produced in the form of NADPH. It is therefore not surprising that the redox state would influence the import of preproteins since proteins should only be imported when there is a demand. Three putative complexes of the TIC translocon have been identified as possible sensors of the chloroplast redox state; Tic32, Tic55 and Tic62 (Figure 1, Calibe et al 1997, Küchler et al 2002, Hörmann et al 2004). All components were first identified in pea. Tic32 was found to be tightly associated with several components of the TIC complex after immunoprecipitation (Hörmann et al 2004). Tic55 and Tic62 were discovered by cross-linking and subsequent blue native PAGE analysis of the TIC complex (Calibe et al 1997, Küchler et al 2002). An example of redox sensitive import was shown for ferredoxin III. Under light conditions the non-photosynthetic ferredoxin III preprotein is transported only as far as the intermembrane space, whereas in the dark complete transfer into the stroma occurs (Hirohashi et al 2001). To date no translocon component has been shown to be responsible for this effect.

Tic62 (Figure 1) shares homology with eukaryotic NAD(P)H dehydrogenases and also with Ycf39-like proteins in cyanobacteria and non-green algae (Table 1, Küchler et al 2002). The Tic62 protein is composed of two structurally different domains as deduced from CD measurements (Stengel et al 2008). The part known to interact with NAD(P)H lies in the N-terminal part of the protein while the C-terminal region carries a site for ferredoxin-NAD(P^+) oxidoreductase (FNR). The FNR protein is an electron transporter in the thylokoid membrane with a reduction potential between ferredoxin and $NADP^+$ (Stengel et al 2008). Chemicals that interfere with NAD binding or affects the NAD(P)/NAD(P)H ratio were shown to influence the import of leaf specific FNR isoforms suggesting that Tic62 regulates the import process through redox sensing (Küchler et al 2002). The association of Tic62 with the TIC complex and FNR as well as the location of Tic62 are all influenced by the $NADP^+$/NADPH redox state in the stroma (Stengel et al 2008).

Tic32 (Figure 1) behaves as an integral membrane protein and shares homology with short chain dehydrogenase/reductase proteins (Table 1).

Immunoprecipitation experiments found Tic32 to be associated with a number of TIC components e.g. Tic22, Tic40, Tic62 and Tic110. A double mutant of Tic32 could not be established so it is probably embryo lethal (Hörmann et al 2004). An interesting finding was that the import of preproteins was reduced in the presence of calcium or calmodulin inhibitors (Chigri et al 2005). This effect was specific for proteins having a cleavable TP. This calcium regulation of import is thought to occur at the inner envelope membrane or possibly in the intermembrane space with calmodulin working together with a number of TIC components. Using affinity chromatography Tic32 was found to be the dominant inner envelope membrane protein bound to calmodulin and this interaction appeared to be calcium dependent (Chigri et al 2006). Tic32 also has a NADPH dependent dehydrogenase activity and NADPH directly affects the interaction of Tic32 with Tic110. The binding of both NADPH and calmodulin are unique to Tic32 suggesting Tic32 to be involved in sensing and co-ordination of the redox potential with the calcium signal (Table 1, Chigri et al 2006). This mass of data shows that the system is complex and a large number of components are required in order to work properly. Another possibility is that several different TOC complexes may exist separately.

7. Tic55

Identification

As many of the other components of the chloroplast import machinery Tic55 (Figure 1) was first characterized in pea. Analysis by blue native gel electrophoresis revealed that the translocon of the inner envelope membranes consisted of at least six proteins with molecular weights of 36, 45, 52, 60, 100 and 110 kDa, respectively (Calibe et al 1997). Tic110 and Hsp93, identified as components of the protein import apparatus of the inner envelope membrane, were prominent constituents of this complex. The amino acid sequence of the 52 kDa protein, deduced from the cDNA, was found to contain a Rieske-type iron-sulfur cluster and a mononuclear iron-binding site (Table 1, Caliebe et al 1997). In another independent experimental approach, the 52 kDa protein could be co-purified with a trapped preprotein protein in association with the chloroplast protein translocon subunits Toc34, Toc75, Toc86 and Tic110. Together, these results led to the suggestion that the novel 52 kDa protein, named Tic55 due to its calculated molecular weight, is a member of the chloroplast inner envelope protein translocon (Calibe et al 1997).

Relatedness to redox and Tic62 and Tic32

Tic55 belongs to a five-member family of non-heme oxygenases defined by the presence of Rieske and mononuclear iron-binding domains. In addition to Tic55, this gene family includes pheophorbide *a* oxygenase (PAO), chlorophyll *a* oxygenase (CAO), choline monooxygenase (CMO), and a Pchlide-dependent translocon component (PTC52, Gray et al 2004). Both Tic55 and PTC52, also known as atTic55-IV in Arabidopsis (Oreb et al 2006**, Paper II**), have been identified as targets for thioredoxins, a family of small regulatory proteins with a redox-active disulfide bridge (Buchanan and Balmer 2005, Bartsch et al 2008). Tic55 lacks the NADPH dehydrogenases found in Tic62 and Tic32 and also lacks the FNR binding site of Tic62. In this sense Tic55 is not similar to components with a clear redox role which

makes it more difficult to argue for a role in this process. The redox sensing ability of Tic55 is believed to reside in the Rieske iron-sulfur centre (Calibe et al 1997).

Function

The role of psTic55 in chloroplast import was investigated by treating chloroplasts with diethylpyrocarbonate (DEPC), which may act on histidine in the Rieske center (Calibe et al 1997). The rationale behind this was a previous study showing that DEPC inhibits the electron transfer activity of mitochondrial complex III. This occurs by direct ethoxyformylation of the histidine residues involved in forming the Rieske iron–sulfur cluster (Ohnishi et al 1994). Nevertheless, the possibility that other proteins were also affected by DEPC was not ruled out. If the effect is Tic55-specific one would expect the atTic55 mutant to be insensitive to DEPC treatment. To our surprise both chloroplasts from wild-type and Tic55 mutant plants were equally affected in terms of protein import in the presence of DEPC (**Paper II**). These finding are also in line with the fact that no phenotypic variation have been found for *tic55-II* mutant plants compared to wild-type plants (Figure 3, **Paper II**) despite the absence of expression of the Tic55 protein (Figure 4, **Paper II**). To make the matter even more complex other groups have been unable to isolate Tic55 together with the TOC/TIC complexes (Kouranov et al 1998, Reumann and Keegstra 1999). However, Tic55 was identified as a target for thioredoxins, which are ubiquitous small proteins with a redox-active disulfide bridge (Bartsch et al 2008). In chloroplasts, two types of Trxs (f and m) coexist and play central roles in the regulation of the Calvin cycle and other processes (**Paper II**). Interestingly, an enzyme protein involved in glycolysis, triose phosphate isomerase, was found to be affected by the Tic55 mutation (**Paper IV**). Recently two Tic55 homologs have been proposed to exist in Arabidopsis, atTic55-II and AtPTC52. A phylogenetic analysis showed that atTic55-II is an ortholog of the psTic55 protein and that AtPTC52 is a more distant homolog of the two making redundancy between the two components unlikely (**Paper II**). The PTC52 protein was demonstrated as a component specific for the Pchlide-dependent translocation of pPORA in barley where it is thought to be part of a distinct translocon (Bartsch et al 2008). Despite a light-induced decline in the amount of PTC52 in barley, this translocon complex is believed to remain active in chloroplasts (Reinbothe et al 2004). However, the PTC52 mutant in Arabidopsis do not display any phenotype and the import of pPORA is not affected (Figure 5, **Paper II**). The absence of any detectable defect indicates that pPORA is not dependent on PTC52 for import, and further supports the previous suggestion that pPORA is translocated through the general TOC and TIC translocons (Aronsson et al 2000).

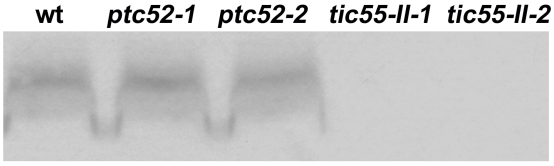

Figure 4. Western blot using a polyclonal antibody raised against psTic55. Aliquots of chloroplast protein samples equivalent to 10 µg protein isolated from 14 day old wild-type, *ptc52*, *tic55-II* plants were separated by SDS–PAGE and then blotted to nitrocellulose membrane. Membranes were blocked using standard procedures, and then incubated with the antibody.

Present research status

It was recently suggested that reduced chloroplast protein import in heat-stressed plants is likely due to decreased gene/protein expression of certain components of the TOC complex (Dutta S et al 2009). The gene expression of Tic20, Tic32, and Tic62 was reduced in heat-stressed samples by 35%, 74%, and 56%, respectively. However, the gene expression of Tic22, Tic40, Tic55, and Tic110 was not significantly affected by high temperatures (Dutta et al 2009). In addition of not being a vital component of redox sensing Tic55 does not appear to be an important mediator of high temperature stress. In fact very little information on the role of Tic55 has emerged since the first publication by Calibe et al and the role as a redox sensor is still vague and under question (**Paper II**). Nevertheless, Tic55 is a verified target for thioredoxis and is thus under redox control (Bartsch et al 2008). If Tic55 acts as a redox sensor it is probably a member of a non-essential redox sensing class of proteins and may act as "fine-tuner" of import at the TIC translocon (Boij et al 2009). The fact that multiple substratesseveral preproteins were imported at normal rates in the Tic55 mutant makes it difficult to proceed with research of Tic55 with an import focus and other tools are needed to elucidate the function of Tic55 (**Paper IV**).

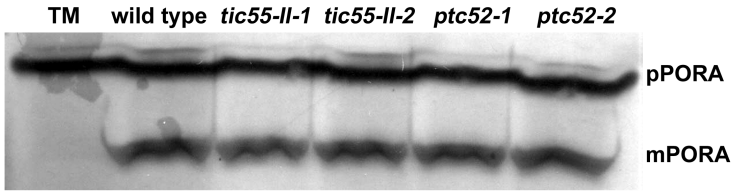

Figure 5. Chloroplast protein import experiment with the precursor of NADPH:Pchlide oxidoreductase (pPORA) using wild-type, *tic55-II* and *ptc52* plants .Chloroplast isolations and import reactions were performed as described by Aronsson and Jarvis (2002). Import was carried out in white light (100 µmol m^{-2} s^{-1}) at 25°C for 10 minutes. TM, translation mixture; pPORA, preprotein of PORA; mPORA, mature PORA. Two separate mutant lines were used for *tic55* and *ptc52* mutants.

PTC52 was suggested as a specific import protein for pPORA by Reibothe et al in 2004 and this hypothesis have not been questioned by other groups. Thus, it was somewhat surprising that the Arabidopsis *ptc52* mutants were indistinguishable from wild-type plants. The lack of an effect of *ptc52* on pSS import was expected since PTC52 has proposed specificity for pPORA (Bartsch et al 2008) (**Paper II**). However, when we incubated the *ptc52* mutant chloroplasts with Arabidopsis pPORA, import rates were found to be normal (Figure 5, **Paper II**). This might be explained by the fact that PTC52 is apparently more active in etiolated plants; however, under light conditions, PTC52 is said to remain active, although at a reduced level. With this in mind a significant effect of PTC52 mutation on the amount of pPORA import should have been detectable (**Paper II**). It may be that PTC52 has specificity for pPORA but since pPORA can be imported normally through the TIC/TOC machinery it is difficult to envision why such a redundancy should be needed even though PORA can be considered a key component for chloroplast development (Aronsson et al 2000).

8. Conclusions and future perspectives

During the last decade substantial progress have been made in the field of chloroplast protein import. Most components of the core complex have been identified and several interaction partners have also been characterized. However, many important questions remain unsolved in order to fully understand every aspect of the import process. The most significant development has been the realization that the targeting of preproteins to chloroplasts is not as simple as was once thought (Aronsson and Jarvis 2008, Kessler and Schnell 2009, Balsera et al 2009). It is probable that multiple mechanisms operate to ensure that the many different proteins that are transported to the chloroplast arrive at the correct location. Whenever no clear function can be ascribed to a specific component it is always tempting to say that it has a "non-essential" role or a "fine tuning" function. There is an inherent risk in this since proteins that are not components of a specific system may be falsely included. The Toc64 protein is without no doubt in the vicinity of the core TOC components since several independent cross-linking experiments have revealed the presence of this protein (Sohrt and Soll 2000, Becker et al 2004a). However, the absence of a detectable phenotype and the lack of impact on import of preproteins clearly argue against a central role in chloroplast protein import (**Paper I**). The component should therefore be renamed as Outer Envelope Protein 64 kDa (OEP64) giving it a more general designation. Data supporting a role for Toc64 as a receptor for chloroplast import are generated biochemically whereas the data arguing against this role were derived in vivo (Hofmann and Theg 2005b, **Paper I**). Since no phenotype could be attained at both plant and chloroplast level the work somewhat halted at this stage and we turned or attention towards the two remaining Toc64 homologues in Arabidopsis Toc64-I and Toc64-V. It is interesting that Toc64-I seems to have a role in auxin biosynthesis (Pollmann et al 2006) since a root growth phenotype that could possibly be attributed to altered auxin levels was found for the Toc64-I mutant (**Paper III**). Furthermore, scrutiny of the mutant lines will reveal if the auxin level is to be altered in vivo. An experiment to discern whether Toc64-V will be able to functionally replace Tom70 in a yeast mutant should be conducted. If Toc64-V can be confirmed as a TPR-receptor in mitochondria this raises the subject of Toc64-III as a receptor at the chloroplast envelope (Mirus et al 2009).

The Tic55 component proved to be even more elusive since no clear task has been suggested beyond the rather fuzzy "redox sensing" function (Bartsch et al 2008). With two other identified redox sensors, Tic62 and Tic32, it is difficult to see why a third protein should have the same function (**Paper II**). Initially it was encouraging to find that there was a second Tic55 homolog in Arabidopsis since functional redundancy could not be ruled out. However, Tic55-II is the true homolog of psTic55 and Tic55-IV (PTC52) was at the same time suggested to constitute a separate translocon (Bartsch et al 2008). PTC52 may still constitute a separate translocon but its role for substrate dependent import of PORA has been questioned (**Paper II**). Further research is needed to fully understand the role of these two components and their possible links to redox control.

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11. Populärvetenskaplig sammanfattning på svenska

Cyanobakterier kan anses vara en primitiv förfader till den moderna kloroplasten och numera anser man att kloroplasten troligen uppkommit genom endosymbiont-teorin. Denna händelse gav kloroplasten fördelen av fotosyntes medan den frilevande cyanobakterien blev skyddad och ökade sina chanser till överlevnad. Från början hade endosymbionten ett eget genom men dessa gener har sedan flyttats till värdcellens genom. Detta ger möjligheter till större kontroll över genuttryck men resulterar samtidigt i en del problem. Man beräknar att ca 95 % av kloroplastens gener numera finns i cellkärnan. Samtidigt så har evolutionen sett till att den ursprungliga cyanobacterien blivit mera komplex genom att en hel del nya gener tillkommit. De gener som transkriberas i cytosolen till mRNA (ritningen för att skapa ett protein) bildar proteiner på ribosomer i cytosolen. Dessa färdiga proteiner måste sedan på något vis ta sig in i kloroplasten genom att passera över biologiska membran. Denna process kallas för protein import och är ett område där en hel del forskning bedrivits. Det är lätt att förstå intresset bakom detta då kloroplastens reaktioner och funktioner är av yttersta vikt inte minst från ett födoperspektiv. Även om huvuddragen i protein-import till kloroplasten är kända så finns det fortfarande en del oupptäckta aspekter och dessutom är funktionen av vissa komponenter för import föremål för debatt. Två av dessa omstridda komponenter är Toc64 och Tic55. Toc64 är föreslagen som en receptor i kloroplastens yttermembran medan Tic55 är föreslagen som en sensor för kloroplastens energistatus. I min forskning har jag undersökt muterade växer som är defekta i produktionen av dessa proteiner. Slutsatsen är att Toc64 troligen representerar en receptorklass som i bästa fall är icke essentiell. Detta bekräftades i huvudsak med hjälp av importförsök där importhastigheten av radioaktivt inmärkta protein undersöktes. Ett protein som är nära släkt med klorplastens Toc64 kan dock vara involverat i biosyntes av växthormonet auxin. Vidare så kunde någon funktion för Tic55 inte fastställas. Plantan och kloroplasten klarar sina funktioner utmärkt utan ett fungerande Tic55 protein. Detta innebär att Tic55 troligen inte är en absolut nödvändig reglerare av importen utan snarare en finreglerare. Ytterligare studier krävs för att kartlägga alla aspekter av protein-import till kloroplasten.