Protein import into plastids

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Abstract

Most chloroplast proteins are synthesized as preproteins with N-terminal transit sequences and imported from the cytosol. Protein translocons at the outer (Toc) and inner membranes of the chloroplast (Tic) recognize the presequence and enable the transfer of the polypeptide across the dual membrane envelope. After the initial characterization of the translocon components, research focused on the import mechanisms and their regulation. Recent research taking advantage of the *Arabidopsis* system has demonstrated the existence of substrate specific import sub-pathways in the general import pathway. New discoveries indicate that glycosylated proteins may take an entirely different pathway via the endoplasmic reticulum and the Golgi apparatus. This review will discuss the known import components in the light of the exciting new discoveries.

1 Plastids

Plants are characterized by a family of double-membrane bound organelles called plastids. Plastids and mitochondria originate from endosymbiotic events. In the case of the plastid, an ancient photosynthetic cyanobacterium was engulfed by a eukaryotic host (McFadden 2001; Kutschera and Niklas 2005). During evolution the plastid retained its own genetic system but most of its genes were transferred to the host cell nucleus (Martin et al. 2002; Leister and Schneider 2003; Timmis et al. 2004). Moreover, the plastid evolved into a family of remarkably versatile organelles (Lopez-Juez and Pyke 2005). Functionally specialized plastid types having varying morphologies and physiological properties are controlled by the host tissue. All plastid types derive from an undifferentiated plastid called proplastid which is present in meristematic cells. Differentiation is achieved by the import of functionally specific sets of protein. The development and interconversion of plastids may also be influenced by environmental cues such as light in the case of chloroplasts.

The chloroplast, present in green aerial tissues, constitutes the site of photosynthesis and metabolic functions such as fatty acid and amino acid biosynthesis or nitrite and sulphate reduction. Chloroplasts contain a unique internal membrane system, the thylakoids harbouring the photosynthetic machinery.

Chloroplasts may differentiate into chromoplasts colouring fruits and flowers due to their accumulation of carotenoid compounds. Finally, amyloplasts, elaio-

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plasts and leucoplasts are unpigmented plastid types present in non-green tissues and generally specialize in the storage of compounds such as starch, lipid, and protein.

1.1 Plastid biogenesis

The biogenesis of the different plastid types is linked to the differentiation of the host cell and is evident in plastid type specific proteomes (Kleffmann et al. 2004; van Wijk 2004; Siddique et al. 2006). Plastids being semiautonomous are largely under genetic control by the host cell and most plastid proteins are encoded in the nucleus. Therefore plastids import proteins that are synthesized in the cytosol. This requires the existence of mechanisms that reliably, specifically and efficiently target and translocate proteins into plastids. In this review, we will give an overview of the main import pathway across the two envelope membranes of chloroplasts as well as a short discussion of alternative import pathways.

2 Chloroplast targeting signals

Most chloroplast targeted proteins are synthesized in the cytosol as precursor proteins with a cleavable, N-terminal targeting signal termed transit sequence (transit peptide). The transit sequence is recognized by chloroplast import receptors and enables the passage of the precursor protein through the import complexes at the outer and inner envelope membrane (Bruce 2000, 2001). Upon translocation, the transit peptide is removed by a stromal processing peptidase yielding the mature protein (Richter and Lamppa 2003). Proteins destined for the thylakoid membrane system of chloroplasts are often synthesized with bipartite signals consisting of an N-terminal transit peptide, for import into the chloroplast stroma, followed by an additional targeting sequence specifying either insertion into the thylakoid membrane or translocation into the thylakoid lumen. At least four pathways contribute to thylakoid targeting and are conserved from the prokaryotic ancestor of the chloroplast: 1) The SRP-pathway facilitates insertion of hydrophobic proteins into the thylakoid membrane. 2) The Sec-pathway promotes translocation into the thylakoid lumen. 3) The TAT-pathway allows for translocation of folded proteins associated with cofactors into the thylakoid lumen. 4) Finally, some proteins may integrate into the thylakoid membrane without assistance by other proteins or energy consumption via the spontaneous insertion pathway. The detailed description of thylakoid translocation and membrane insertion exceeds the scope of this paper and we recommend the lecture of one of the excellent reviews on the subject (Jarvis and Robinson 2004; Gutensohn et al. 2006).

2.1 Structure of transit peptides

Transit peptides typically have 20 to > 70 amino acids. They are rich in hydrophobic and hydroxylated residues and have few acidic amino acids resulting in a net positive charge. The entire transit peptide seems to be necessary for correct targeting (Bhushan et al. 2006). No conserved sequence motifs or secondary structure elements have been identified complicating the definition of common features for chloroplast targeting. In aqueous environments transit peptides have been shown to be largely unstructured and form a random coil (Bruce 2001). However transit peptides may undergo secondary structural changes upon interaction with lipids (Horniak et al. 1993) or with receptor components (Bedard and Jarvis 2005) possibly corresponding to molecular events taking place during the import process. Transit peptides interact with Hsp70 molecular chaperones (Ivey and Bruce 2000; Rial et al. 2000; Zhang and Glaser 2002). Binding to cytosolic Hsp70s most likely prevents precursor protein aggregation prior to import, whereas binding to Hsp70 in the course of import might facilitate the translocation process.

3 Energy requirements of *in vitro* chloroplast protein import

Biochemical experiments carried out with isolated chloroplasts from pea (*Pisum sativum*) revealed the energy-requirements of chloroplast protein import and resulted in a three step model of protein import (Fig. 1) (Schnell and Blobel 1993). In the first step the precursor protein is recognized at the outer envelope membrane.

3.1 Precursor protein recognition at the chloroplast surface

This initial binding of a precursor protein to receptor components at the chloroplast surface does not require energy and is reversible (Perry and Keegstra 1994). In the second step, irreversible binding of the precursor protein at the outer membrane occurs.

3.2 The early translocation intermediate

Irreversible binding of the precursor protein at the outer membrane requires GTP as well as low concentrations of ATP ($\leq$ 100 μM) in the intermembrane space (Olsen and Keegstra 1992; Kessler et al. 1994; Young et al. 1999). The outer membrane-bound form has been termed the “early intermediate” (Schnell and Blobel 1993). At the early intermediate stage the precursor protein extends across the outer membrane and makes contact with protein components at the surface of the inner membrane (Wu et al. 1994; Ma et al. 1996). 100 μM ATP may promote

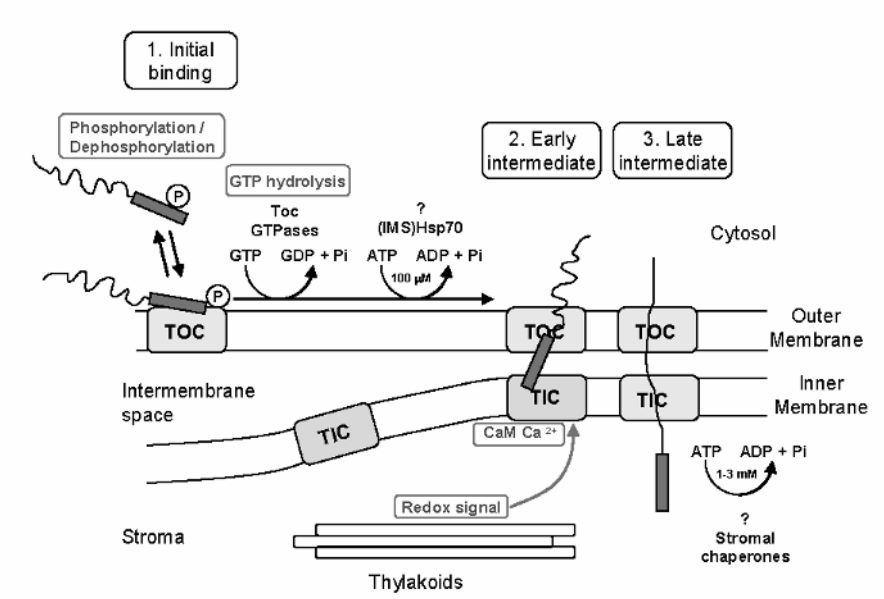


Fig. 1. Stages and regulation of Toc/Tic mediated chloroplast protein import. The three stages (1.-3.) were defined based on the energy requirements of *in vitro* import into isolated chloroplasts. 1. The initial binding of the precursor protein to the chloroplast surface does not require energy and is reversible. 2. For irreversible binding and partial translocation GTP and low amounts of ATP are required, probably used up by the Toc GTPases and intermembrane space-located Hsp70s, respectively. 3. Completion of translocation requires higher concentrations of ATP in the stroma, presumably consumed by stromal chaperones at the inner surface of the envelope. Phosphorylation/dephosphorylation of the transit-peptides and the Toc GTPases as well as GTP-hydrolysis regulate the Toc complex, whereas the Tic complex is regulated by redox-signals and calcium-calmodulin.

precursor protein binding to an intermembrane space Hsp70 protein preventing re-exit into the cytoplasm (Schnell et al. 1994; Becker et al. 2004b).

3.3 The late translocation intermediate

Completion of translocation requires higher concentrations of ATP (1-3 mM) in the stroma (Pain and Blobel 1987; Theg et al. 1989). The ATP in the stroma is presumably consumed by stromal chaperones such as Hsp60 (Cpn60), Hsp93 (ClpC) or of the Hsp70 family at the inner surface of the envelope (Kessler and Blobel 1996). Rapid chilling of the import reaction in the presence of 1-3 mM ATP results in the kinetic trapping of the precursor protein extending across both envelope membranes and engaging the translocation machineries at both the outer and inner envelope membranes. This trapped precursor constitutes the so-called "late intermediate" (Schnell and Blobel 1993).

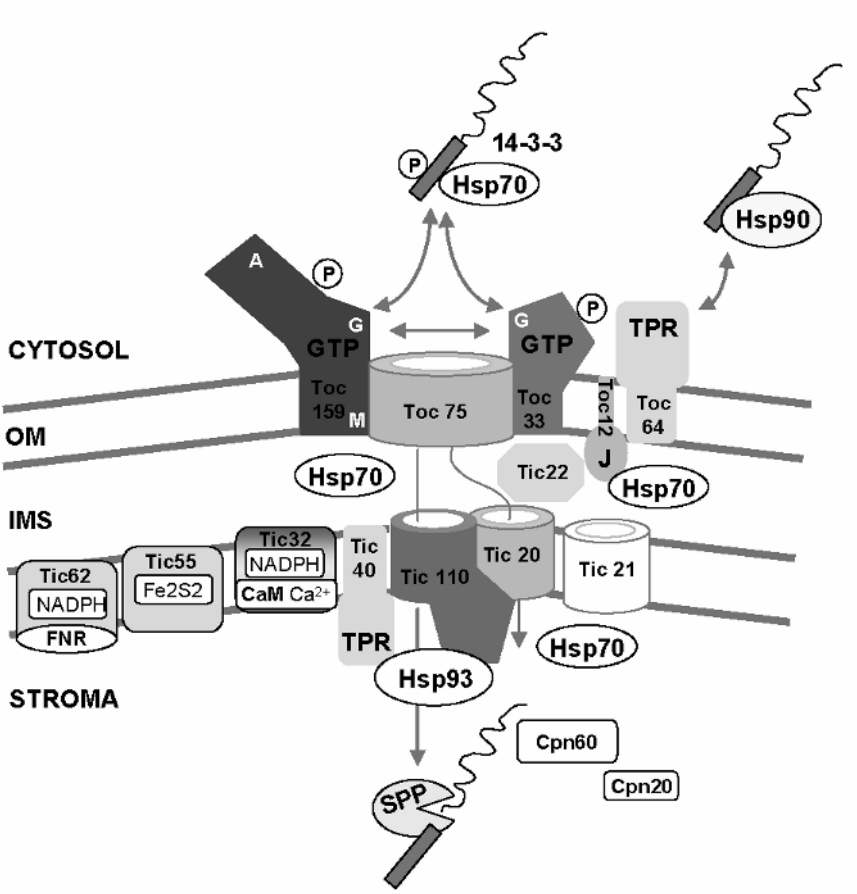
Fig. 2. overleaf. Overview of components of the Toc/Tic chloroplast import machinery of the model plant *Arabidopsis thaliana*. Phosphorylated or non-phosphorylated precursor proteins with an N-terminal transit-peptide are associated with molecular chaperones of the Hsp70 or Hsp90 family and 14-3-3 proteins. They are recognized at the chloroplast surface by the receptor GTPases of the Toc complex (translocase at the outer membrane of chloroplasts), Toc159 and Toc33. Hsp90 associated precursor proteins are recognized by the co-receptor Toc64 before being transferred to Toc33. The GTP-dependent interaction of Toc159 and Toc33 promotes the transfer of the precursor protein to the translocation channel Toc75. In the intermembrane space (IMS), the precursor protein interacts with Hsp70 recruited by the J-domain of the Toc component Toc12 and with Tic22. This interaction probably favour the tight association of the Toc and Tic complexes (translocase at the inner membrane of chloroplasts). Tic110 and/or Tic20 constitute the translocation channel at the inner membrane of the chloroplast. Tic110 has an additional role in the recruitment of chaperones (Hsp93 and cpn60) to the translocation machinery and is assisted by the co-chaperone Tic40. In the stroma the transit-peptide is cleaved off by stromal processing peptidase (SPP) and the folding of the mature protein is aided by the chaperonins cpn60 and cpn20. Tic62, Tic55, and Tic32 are redox-sensing Tic components having a regulatory function. As a calmodulin binding protein Tic32 is also involved in calcium-regulation of the import process. Like Tic20, Tic21 may take over a function as part of the inner membrane protein-conducting channel and replace Tic20 depending on the developmental stage.

4 Identification of components of the translocation machinery

Chloroplast envelope proteins participating in precursor protein import were first identified in the *in vitro* import system using pea chloroplasts. Different experimental approaches using translocation intermediates, chemical crosslinking and antibody inhibition, respectively, resulted in overlapping sets of candidate components (Fig. 2) (Hirsch et al. 1994; Kessler et al. 1994; Perry and Keegstra 1994; Schnell et al. 1994). Interestingly, three of the components now termed Toc159, Toc34, and Toc75 (Translocase at the outer membrane of the chloroplast followed by the molecular mass in kilodaltons) were associated with both the early intermediate and the late intermediate whereas another protein Tic110 (Translocase at the inner membrane of the chloroplast) was exclusively associated with the late intermediate (Schnell et al. 1994). These findings are consistent with the early intermediate extending solely across the outer membrane and the late intermediate extending across both membranes simultaneously engaging components at both envelope membranes.

4.1 Components of the Toc complex

To date five different components of the pea Toc complex have been described: two GTPases, Toc159 and Toc34, and a channel protein Toc75 together form a



stable core complex. In addition, a TPR-domain containing protein Toc64 (Sohrt and Soll 2000) and J-domain containing protein Toc12 (Becker et al. 2004b) have been identified as loosely associated components.

4.1.1 The Toc core complex

Reconstitution experiments into artificial lipid vesicles have demonstrated that the Toc core complex consisting of a 86 kDa proteolytic fragment of Toc159 (Toc86, see also 4.1.2), Toc34 and Toc75 translocates precursor proteins *in vitro* (Schleiff et al. 2003b). Analysis of the Toc core translocon purified from pea, revealed a molecular mass of ~ 500 kDa in size exclusion chromatography and a stoichiometry of Toc159:Toc34:Toc75 of 1:4:4 in this complex (Schleiff et al. 2003a). In single particle electron microscopy of the complex, 13 nm toroid-shaped particles were observed, which appeared in three-dimensional reconstitution as structures with four pores and a central finger domain (Schleiff et al. 2003a). In BN-PAGE the pea Toc complex migrates at 800-1000 kDa (Kikuchi et al. 2006; Chen and Li

2007). The molecular weight differences observed may be due to the techniques employed, partial degradation or the presence of Toc64, Toc12, precursor proteins or so far unidentified components in the higher molecular weight complex.

4.1.2 Toc159 and Toc34: chloroplast protein import receptors

Toc159 and Toc34 are protease sensitive proteins in isolated chloroplasts indicating exposure at the chloroplast surface (Hirsch et al. 1994; Kessler et al. 1994; Seedorf et al. 1995). In agreement with the finding numerous studies indicate a role in precursor protein recognition. The two proteins have homologous GTP-binding domains (G-domains). GTP-dependence of early intermediate formation implicates the two proteins in the early stages of protein import. Toc159 has an additional N-terminal acidic domain (A-domain) and is anchored in the outer chloroplast membrane by a C-terminal membrane anchoring domain (M-domain). Due to the protease sensitivity of the A-domain, pea Toc159 was initially identified as a smaller protein designated Toc86 comprising only the G- and M-domains (Bolter et al. 1998). In addition to the membrane-integrated form, a portion of cellular Toc159 was present as a soluble, cytoplasmic protein, hinting at the dynamics in Toc complex assembly (Hiltbrunner et al. 2001b; Bauer et al. 2002; Smith et al. 2002). Toc34 consists of the G-domain followed by a stretch of hydrophobic amino acids anchoring the protein in the outer membrane. The crystal structure of pea Toc34 showed GDP-bound dimers (Sun et al. 2002). Biochemical studies indicate that Toc34 not only forms homodimers but may also form heterodimers with Toc159 via their respective GTP-binding domains (Weibel et al. 2003).

4.1.3 Toc75: the protein conducting channel

Toc75 has similarity to bacterial solute channels and was therefore identified as a candidate for a protein conducting channel (Schnell et al. 1994; Gentle et al. 2005; Schleiff and Soll 2005). Electrophysiological studies on the reconstituted protein demonstrated ion conducting channel properties (Hinnah et al. 1997, 2002). During early intermediate formation the precursor protein is thought to insert across the Toc75 channel and make initial contact with Tic components at the outer surface of the inner membrane. Interestingly, Toc75 has sequence homology with Omp85 in bacteria and Tob55 in yeast which are proteins involved in the insertion of beta-barrel proteins in the bacterial and mitochondrial outer membranes respectively (Gentle et al. 2005; Paschen et al. 2005).

4.1.4 Toc64

Toc64 is loosely associated with the Toc-complex and therefore not considered part of its core (Sohrt and Soll 2000; Qbadou et al. 2006). Toc64 contains TPR repeats forming a putative docking site for a subset of precursor proteins bound to cytosolic Hsp90. The Toc64 TPR domain interacts with the receptor GTPase Toc34 initiating the transfer of the precursor protein to the Toc complex (Qbadou et al. 2006). Its function as an accessory import receptor for a subset of precursor

proteins may be comparable to the function of Tom70 in mitochondrial protein import (Young et al. 2003). Toc64 is non-essential both in *Arabidopsis thaliana* and *Physcomitrella patens*. Moreover, Toc64 deficient *Physcomitrella patens* showed no chloroplast protein import deficiency. Therefore, PpToc64 has been renamed PpOEP64 (Hofmann and Theg 2005b).

4.1.5 Toc12

Toc12 is a J-domain containing protein facing the intermembrane space (Becker et al. 2004b). It stimulates ATP hydrolysis by DnaK *in vitro* and interacts directly with intermembrane space localized Hsp70 proteins. Toc12 may therefore function to anchor an intermembrane space Hsp70 which in turn retains precursor proteins in the translocation channel and prevents them from slipping back into the cytosol. The J-domain of Toc12 may be conformationally stabilized by an intramolecular disulfide bridge, which could be sensitive to the redox state of the chloroplast (Becker et al. 2004b; Bedard and Jarvis 2005).

4.2 Components of the Tic complex

A number of components of the inner membrane translocation machinery have been identified (Fig. 2). Unlike the Toc core complex, they do not appear to form a stable complex.

4.2.1 Tic22 and Tic20

Both Tic22 and Tic20 were not originally identified as components of the early or late translocation intermediates. However, chemical crosslinking at the early intermediate stage revealed the presence of the two inner membrane proteins and indicated that the precursor proteins had completely traversed the outer chloroplast membrane (Kouranov and Schnell 1997; Kouranov et al. 1998; Chen et al. 2002). Biochemical analysis demonstrated that Tic22 was an extrinsic protein exposed at the surface of the inner membrane suggesting a function as an inner membrane receptor or in the formation of contact sites between the import complexes (Kouranov and Schnell 1997; Kouranov et al. 1998).

In contrast, Tic20 was integral to the inner membrane suggesting a function as a component of the protein conducting channel at the inner membrane. Tic20 is predicted to span the membrane with four transmembrane α-helices (Kouranov et al. 1998; Chen et al. 2002) and shares some similarity with TIM channel proteins (Reumann and Keegstra 1999). A role as an inner membrane protein-conducting channel expressed in all tissues and likely to be required for early plastid development has been proposed (Chen et al. 2002; Teng et al. 2006).

4.2.2 Tic110

Tic110 was first described as a component of the late translocation intermediate (Schnell et al. 1994; Wu et al. 1994). The sequence contains two predicted hydrophobic alpha helices at the N-terminus and a large hydrophilic domain at the C-terminus (Kessler and Blobel 1996; Lubeck et al. 1996). Topology data suggest that the C-terminus extends into the stroma (Kessler and Blobel 1996; Jackson et al. 1998; Inaba et al. 2003). The C-terminus, in conjunction with Tic40 functions as a binding site for the chaperones ClpC and Hsp60 which are required for folding subsequent to import (Chou et al. 2003, 2006; Kovacheva et al. 2005). Via its transmembrane helices, Tic110 may participate in the formation of the translocation channel at the inner membrane (van den Wijngaard and Vredenberg 1999; Heins et al. 2002).

4.2.3 Tic40

Tic40 functions at the same late stage of import as Tic110 and Hsp93 (ClpC) and is recruited to Tic110 upon transit-peptide binding by Tic110. It is a membrane-anchored protein with a large hydrophilic domain protruding into the stroma. The C-terminal portion of Tic40 contains similarity to the co-chaperones Sti1p/Hop (Stahl et al. 1999; Chou et al. 2003). Recently, it was demonstrated that Tic40 indeed acts as a co-chaperone by stimulating ATP hydrolysis by Hsp93 and transit-peptide release from Tic110 (Chou et al. 2006).

4.2.4 Tic21

AtTic21 (also known as CIA5) was identified in a genetic screen scoring for mistargeting and accumulation of an antibiotic resistance marker in the cytosol of *Arabidopsis* (Sun et al. 2001; Teng et al. 2006). The phenotype of the knockout of the *Arabidopsis* gene At2g15290 annotated as atTic21 (CIA5) (Teng et al. 2006) was published by two independent research groups recently (Teng et al. 2006; Duy et al. 2007). Both groups found that At2g15290 mutant plants are seedling lethal on soil and chlorotic to albino when cultivated on media supplemented with sucrose. Furthermore, in accordance with each other, both studies identified the corresponding gene product as an integral inner envelope membrane protein of chloroplasts. However, different functions were attributed to the At2g15290 gene product. According to Teng et al. (Teng et al. 2006) At2g15290 (atTic21) functions as a part of the inner membrane protein-conducting channel, similar to Tic20 but at later stages of leaf development. Strong arguments for a function of At2g15290 in chloroplast protein import are the observed inner membrane import defect of chloroplasts isolated from plants expressing a mutated variant of the protein (*tic21*/*cia5* K112C) as well as the co-precipitation of the putative Tic21 with Toc and Tic components even in the absence of crosslinking agent (Teng et al. 2006). In contrast, Duy et al. (Duy et al. 2007) claimed At2g15290 to encode a permease that functions in iron transport across the inner envelope of chloroplasts and therefore annotated the gene as PIC1 for PERMEASE IN CHLOROPLASTS

1 (Duy et al. 2007). Indeed, At2g15290 shares sequence similarity with potential metal iron transporters from cyanobacteria (e.g. *Synechocystis* sll1656). In Affimetrix microarray analysis of the At2g15290 mutant changes in the expression of metal homeostasis-associated and a drastic downregulation of photosynthetic genes were observed (Duy et al. 2007). Ferritin was found to be upregulated and an accumulation of ferritin clusters in plastids was revealed by ultrastructural analysis. It is counterintuitive that the lack of a putative iron permease causes phytoferritin accumulation normally observed as consequence of iron-overload. Expression of the cDNA of At2g15290 as well as of its *Synechocystis* homolog sll1656 in a yeast mutant defective in low- and high-affinity Fe uptake partially restored its growth defect under iron-limited conditions (Duy et al. 2007) indicating their ability to transport iron. However, over-expression of *Synechocystis* sll1656 in the *Arabidopsis* At2g15290 knockout mutant did not result in complementation (Teng et al. 2006). Therefore At2g15290 is most likely not just an "ancient" permease but acquired additional essential functions during evolution. Additional work is clearly required to exclude a role of At2g15290 in chloroplast protein import.

4.2.5 Tic55

Tic55, Tic62, and Tic32 have been identified as a redox-sensing Tic components. Tic55 is an integral protein at the inner membrane of chloroplasts and comigrates with Tic110 and ClpC in BN-PAGE (Caliebe et al. 1997). Tic55 contains a Rieske-type iron-sulphur cluster and a mononuclear iron binding site and may therefore catalyse electron-transfer reactions. Tic55 has been suggested to act as regulatory component of the Tic complex involved in signal-transduction or redox-regulation during protein import (Soll 2002).

4.2.6 Tic62

Tic62 has a conserved NAD(P) binding site at its N-terminus and a binding site for ferredoxin-NAD(P) reductase (FNR) at its stroma-exposed C-terminus (Kuchler et al. 2002). The photosynthetic electron flux may regulate the import apparatus via FNR, transferring electrons from ferredoxin to the NAD(P) associated with Tic62.

4.2.7 Tic32

Tic32, with similarity to short-chain dehydrogenase/reductase (SDR), is an integral inner envelope protein and was shown to associate with Tic110 (Hormann et al. 2004). Tic32 is an essential protein, the *tic32 Arabidopsis* knockout mutant being embryo lethal (Hormann et al. 2004). Notably, Tic32 may function not only in redox but potentially also in calcium regulation of the protein import (see section calcium regulation) (Chigri et al. 2006). A striking feature of Tic32 is its lack of a cleavable transit-peptide and its import by a so far unknown pathway (Nada and Soll 2004) (see section alternative import pathways).

5 Regulation at the Toc and Tic complexes

The nature of some of the Tic and Toc proteins hint at their regulation. Analysis of GTP-binding proteins Toc34 and Toc159 in the Toc-complex has demonstrated their regulation by GTP-binding and hydrolysis. Phosphorylation/dephosphorylation of some precursors and Toc-components may provide an additional layer of regulation at the Toc-complex. At the Tic complex. Tic55, Tic62, and Tic32 suggest regulation by the redox state of the chloroplast and calcium signalling (Fig. 1).

5.1 GTP-regulated protein recognition at the Toc complex

GTP-binding and hydrolysis at the Toc receptor GTPases Toc159 and Toc34 most likely explain GTP-dependent precursor binding to the chloroplast surface as well as formation of the early import intermediate. Toc159 and Toc34 share some motifs involved in GTP-binding and hydrolysis with Ras-like GTPases (Kessler et al. 1994; Sun et al. 2002). However, the crystal structure of pea Toc34 (psToc34) revealed significant structural variations when compared to Ras-like GTPases. This suggests that the Toc-GTPases utilize a unique mechanism of GTP binding and hydrolysis (Sun et al. 2002) and therefore constitute a new class of GTPases. The observation of GDP-bound Toc34 homodimers as well as Toc159/Toc34 heterodimers suggests that the early stages of import involve GTPase-regulated interactions of the Toc-GTPases (Smith et al. 2002; Sun et al. 2002). In pea, Toc159 and Toc34 are the only Toc-GTPases known so far, but in other species small families have been identified: in *Arabidopsis thaliana* Toc159 has four homologues (atToc159, -132,-120 and -90) and Toc34 has two (atToc33 and Toc34). AtToc159 and atToc33 are considered the orthologs of pea Toc159 and Toc34, respectively.

5.1.1 Toc GTPase cycle

Small GTPases are known to behave like molecular switches cycling between "active" GTP-bound and "inactive" GDP bound states (Bourne et al. 1990). This normally involves interactions with regulatory proteins such as guanine nucleotide exchange factors (GEFs) (Cherfils and Chardin 1999) or GTPase activating proteins (GAPs) (Scheffzek and Ahmadian 2005). To date no GEFs for the Toc GTPases have been identified. But the low intrinsic GTP hydrolysis rate of Toc34 was stimulated by precursor proteins that may therefore serve as GAPs (Jelic et al. 2002; Becker et al. 2004a).

The crystal structure of Toc3, revealed a GDP-bound homodimer. The arrangement of the two Toc34 monomers suggested that one could act as a GAP for the other, by inserting a catalytic residue (arginine 133 in psToc34, arginine 130 in atToc33) into the active site of the other (Sun et al. 2002). However, studies diverge on the catalytic constants of the GTPase activities of the psToc34(R133A)

and atToc33(R130A) mutants with regard to the wild type (Weibel et al. 2003; Reddick et al. 2007; Yeh et al. 2007). This has been attributed to the differing experimental conditions used by the different groups. As a consequence a clear verdict for or against the arginine finger theory is not yet possible.

In addition to its proposed function as an arginine finger, Arg133 of pea Toc34 (Arg130 in *Arabidopsis* Toc33) plays a key role in homodimer formation as well as heterodimer formation with Toc159. The mutated proteins psToc34(R133A) as well as atToc33(R130A) behave as monomeric proteins (Weibel et al. 2003; Reddick et al. 2007; Yeh et al. 2007) and are reduced in their ability to interact with Toc159 (Weibel et al. 2003). Recently, the crystal structure of atToc33(R130A) was published (Yeh et al. 2007). The atToc33(R130A) mutant indeed crystallized as a monomer its structure strongly resembling the monomer structure of psToc34 (Sun et al. 2002). The crystal structure of wild type atToc33, which would be valuable for the evaluation of the structural changes caused by the R130A mutation, is not available. It now appears clear that the dimerization between the Toc-GTPases and their respective interactions with precursor proteins are intimately entwined with GTP-binding and -hydrolysis. Many of the details including the existence of Toc34 homodimers *in vivo* and the nature of nucleotide exchange factors are still mysterious.

5.1.2 Interplay of the Toc GTPases

Substantiated by a series of studies it is well established that Toc159 and Toc34 bind to precursor proteins and dimerize via their GTP binding domains (Kessler and Schnell 2002, 2004). Regardless, the order of the GTP-regulated import events *in vivo* is not known with certainty and has been obscured by the unexpected complexity of the system.

One possibility is that the targeting of a cytosolic precursor protein to the Toc complex is coupled to the GTP-dependent association of the either soluble or integral membrane Toc159 receptor with the Toc-complex (Bauer et al. 2002; Smith et al. 2002; Bedard and Jarvis 2005). In this scenario, termed the targeting hypothesis, the precursor protein in complex with Toc159 is targeted to the Toc complex, where a homotypic interaction between Toc159 and Toc34 coupled to GTP hydrolysis initiates the association of Toc159 in the Toc complex and the transfer of the precursor into the translocation channel Toc75.

The observation that Toc159 GTPase mutants affected in GTP-binding and/or hydrolysis are mislocated to the cytosol and fail to functionally complement the import defect of the *toc159* null mutant *ppi2* (see section 6.1.1) are compatible with this hypothesis (Smith et al. 2002; Lee et al. 2003). The minimal requirements for Toc159 insertion were studied in an *in vitro* system with purified Toc core components reconstituted into artificial vesicles (Wallas et al. 2003). The study demonstrated that the insertion of Toc159 does not only require GTP hydrolysis at both GTPases as well as the presence of Toc75 but also involves a previously unknown interaction of the Toc159 membrane anchoring M-domain with the G-domain of atToc33 (Wallas et al. 2003). Upon insertion into the Toc complex the membrane anchoring domain appears to assume a substantial role in the

import reaction itself. The fact that proteolytic removal of the cytosol-exposed A- and G-domain of Toc159 did not completely deactivate pre-protein import *in vitro* (Chen et al. 2000a) and that the M-domain alone partially complements the *ppi2* mutant (Lee et al. 2003), indicates that the M-domain is more than a passive membrane-anchor and may participate in translocation channel formation.

According to motor hypothesis likewise founded on a series of studies (Schleiff et al. 2003a, 2003b; Becker et al. 2004a), Toc159 functions only after membrane integration as a GTP-driven motor protein at the centre of the Toc complex. This motor hypothesis is based on an *in vitro* reconstitution experiment in which the carboxy-terminal Toc86 fragment of pea Toc159 alone was sufficient to mediate GTP-dependent translocation of a precursor across the Toc75 translocation channel (Schleiff et al. 2003b).

The motor hypothesis proposes that Toc34 acts as an initial receptor and Toc159 as a docking partner for recruiting Toc34. The transit-peptide of the precursor stimulates an interaction of two GTPases in their GTP-bound state. GTP-hydrolysis at Toc34 results in the transfer of the transit peptide to Toc159 and dissociation of Toc34 from the complex. Subsequent cycles of GTP-hydrolysis at Toc159 push the precursor protein across the translocation channel.

The two hypotheses have the GTP-regulated precursor recognition and the interaction of Toc159 and Toc34 in common. They differ mainly in the hierarchy of the two Toc GTPases, which has proven difficult to resolve. Further investigation will be required to determine the mechanistic details of the Toc GTPase cycle. For example, how the crucial GDP-GTP nucleotide exchange occurs at Toc159 and Toc34 is completely enigmatic so far.

5.2 Regulation by phosphorylation

Phosphorylation of the transit peptide of the small subunit of Rubisco in the cytosol has been demonstrated to influence the rate of its import *in vitro* (Waegemann and Soll 1996; May and Soll 2000). Transit peptide phosphorylation permits the binding of cytosolic 14-3-3 proteins and Hsp70 molecular chaperones. This complex of 14-3-3 proteins and Hsp70s has been designated the guidance complex and was shown to stimulate import three to fourfold (May and Soll 2000). Moreover, phosphorylation influences the recognition of the precursor proteins by the import receptors, as Toc34 binds with high affinity to phosphorylated precursors (Sveshnikova et al. 2000) whereas Toc159 recognizes only non-phosphorylated precursors (Becker et al. 2004a). Mutating the phosphorylation site of precursor proteins does not result in mistargeting *in vitro* and *in vivo* (Nakrieko et al. 2004). Thus, transit peptide phosphorylation is not essential for targeting specificity but influences import kinetics. A serine/threonine-specific protein kinase activity for transit peptides was found in pea leaf mesophyll cells and wheat germ lysate (Waegemann and Soll 1996; May and Soll 2000) and recently a family of chloroplast precursor protein kinases was purified from *Arabidopsis* (Martin et al. 2006). The latter consists of three cytosolic serine/threonine kinases (At2g17700,

At4g35780, At4g38470). The three kinases utilize ATP to phosphorylate several chloroplast precursors but not a mitochondrial precursor protein *in vitro*.

Not only precursor proteins but also the Toc GTPases (Toc159 and Toc34 of pea) are subject to phosphorylation (Sveshnikova et al. 2000; Fulgosi and Soll 2002). Two outer envelope kinases phosphorylating the two receptors were partially purified from pea chloroplasts (Fulgosi and Soll 2002). Phosphorylation of pea Toc34 at serine 113 and atToc33 at serine 181 was demonstrated to inhibit precursor protein recognition and GTP binding *in vitro* (Sveshnikova et al. 2000; Jelic et al. 2002; Jelic et al. 2003). Mutations in atToc33 that prevent or mimic phosphorylation at serine 181 did not influence the function of the receptor *in vivo* (Aronsson et al. 2006). In summary, phosphorylation of the import receptor Toc33, similar to precursor protein phosphorylation, is not essential *in vivo*, but may influence the rate of import, which is consistent with a regulatory function.

5.3 Redox-regulation

The redox-state of the chloroplast depends on light and consequently photosynthetic electron transport. It has been suggested to have a regulatory influence on chloroplast protein import (Caliebe et al. 1997; Kuchler et al. 2002; Hormann et al. 2004). Under illumination, the reducing power could act on the redox-sensing components of the Tic complex Tic55, Tic62, and Tic32 as well as on the disulphide bridge stabilizing the J-domain of Toc12 and thereby modulate the import characteristics of the Toc and Tic complexes.

Support for a role of the chloroplast redox state in the regulation of chloroplast protein import came from studies on precursors that exhibit distinct import patterns in the chloroplast under light and dark conditions (Hirohashi et al. 2001). The precursors of maize FdIII (non-photosynthetic ferredoxin) and FNRII (ferredoxin-$NADP^+$ reductase II) accumulated in the intermembrane space of the chloroplast envelope membranes, whereas in the dark, the proteins were processed correctly. Furthermore import experiments with NAD-analogues indicated that the precursor of one isoform of *Arabidopsis* leaf specific FNR (pFNR-L1) is translocated preferentially at a high NAD(P)/NAD(P)H ratio, i.e., in the dark (Kuchler et al. 2002). As many precursor proteins are imported into chloroplasts equally well in the light as in the dark, the light-/redox-regulation is probably not a general regulatory element of the Toc and Tic complexes but rather specific for certain precursor proteins.

5.4 Calcium/calmodulin regulation

Recently, a new mode of regulation of chloroplast protein import by calcium has been proposed (Chigri et al. 2005, 2006). Ophiobolin A, a specific inhibitor of calmodulin, as well as two calcium ionophores inhibited the import of precursor proteins with N-terminal cleavable presequences into isolated pea chloroplasts. The calcium regulation seems to be restricted to the Toc/Tic import pathway, as

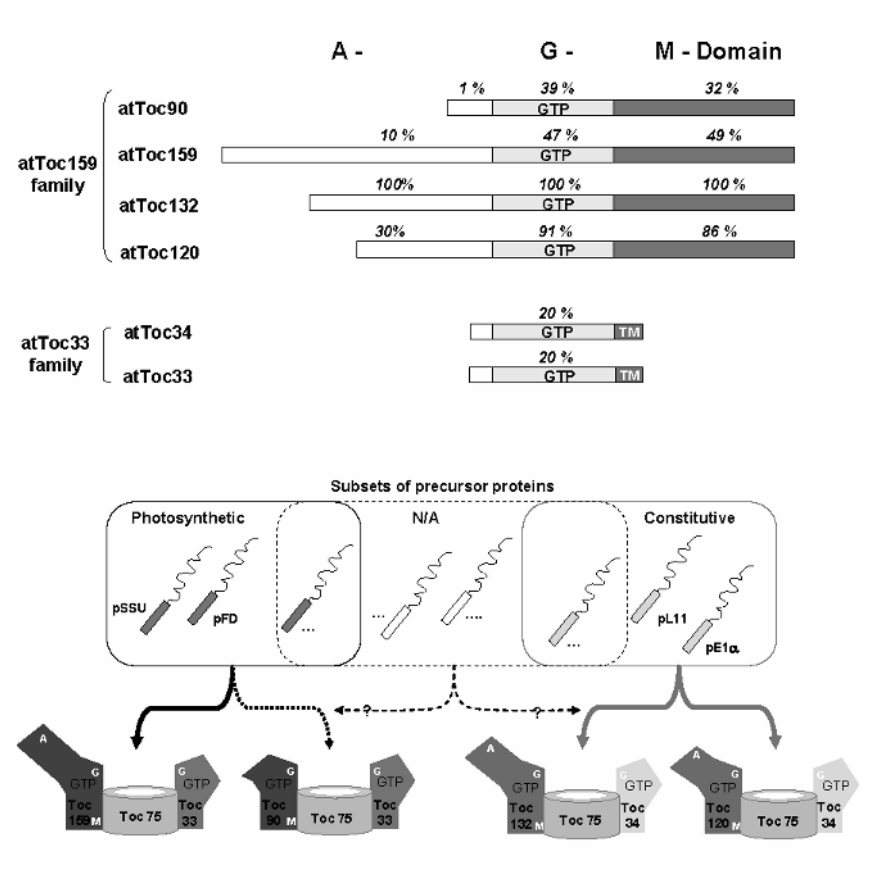


Fig. 3. The Toc GTPase families of *Arabidopsis thaliana*. The upper part shows a linear representation of the four Toc159 homologues (Toc90, Toc159, Toc132, and Toc120), and the two Toc34 homologues (Toc33 and Toc34) including the position of the acidic domains (A), GTPase domains (G) and membrane-anchoring domains (M). Toc33 and Toc34 consist only of a GTPase domain and a small trans-membrane anchor (TM). The values above the domains give the percentage of sequence identity between the domains relative to Toc132. The borders of the G-domains for this analysis were defined as follows: Toc90 aa 38-399, Toc159 aa 727-1092, Toc132 aa 455-805, Toc120 aa 339-687. Genetic and biochemical studies suggest that the members of the Toc GTPase families assemble into different Toc complexes with different substrate specificities (lower part). Toc159 associates preferentially with Toc33 to form the main import complex for photosynthetic proteins. Toc132 and Toc120 associate preferentially with Toc34 into import complexes more involved in the import of constitutive house-keeping proteins. The determinants in the transit-peptides for a specific import pathway have not yet been defined, therefore, a clear classification of precursor protein subsets is not possible. Toc90 may have a minor role in photosynthetic protein import. A preferential association with Toc33 has not been demonstrated.

the import of proteins that do not contain a presequence like psToc34 and psToc32 was not affected (Chigri et al. 2005). The site of ophiobolin A action could be located at the inner envelope. Intriguingly, Tic32, a component of the Tic complex, turned out to be the only calmodulin-binding protein at the inner envelope membrane (Chigri et al. 2006). As Tic32 also has NADPH-dependent dehydrogenase activity it could act as a transducer of both redox and calcium regulation of chloroplast protein import. It has been proposed that the association of Tic32 with other Tic components may be regulated in a NADPH and/or Ca^{2+}/calmodulin dependent manner (Chigri et al. 2006). A number of candidate chloroplast calmodulin and calmodulin-like sequences with potential N-terminal transit sequences have been retrieved from the databases (Chigri et al. 2005). Currently, the nature of the chloroplast calmodulin or calmodulin-like proteins involved in import regulation is not known.

6 Functional specialization in the general import pathway

In *Arabidopsis* the chloroplast protein import receptors pea Toc34 and Toc159 are encoded by small gene families of two (atToc33 and atToc34) and four genes (atToc159, atToc132, atToc120, atToc90), respectively (Fig. 3) (Jackson-Constan and Keegstra 2001a). All Toc34/Toc159 homologous in *Arabidopsis* share sequence similarity in their central GTP-binding domain and seem to derive from an ancient eukaryotic GTP-binding protein that evolved into the Toc GTPases and the AIG family of GTPases (avirulence induced gene) (Reuber and Ausubel 1996; Reumann et al. 2005). All Toc159 homologues have C-terminal membrane anchoring domains (M-domains) but differ significantly in their N-terminal parts. Toc90, the most ancient and distant family member lacks the N-terminal acidic domain (A-domain) and instead has only a short non-acidic N-terminal extension. The other members atToc159, atToc132, and atToc120 as a consequence of domain enlargement and introduction of negative charges have A-domains that vary greatly in length and sequence between the different isoforms. Phylogenetic analysis clearly revealed that atToc132 and atToc120 form a subgroup in the Toc159 family (Hiltbrunner et al. 2001a; Kubis et al. 2004). There is increasing evidence that the members of the Toc GTPase families represent functionally specialized import receptors assembling into Toc complexes of distinct composition. This model is supported by expression patterns of the isoforms and phenotypes of mutants for these receptors as well as complementation studies (Hiltbrunner et al. 2001a; Ivanova et al. 2004; Kessler and Schnell 2004; Kubis et al. 2004).

6.1 Plastid protein import mutants and phenotypes

Plastid protein import mutants exhibit a gradient of phenotypes ranging from embryo lethal to wild type (Table 1). The analysis of import mutants has provided important new insight into the import process and its role in plant development.

Table 1. Summary of *Arabidopsis* homologues of the pea chloroplast import machinery including single mutant phenotypes

Pea	*Arabidopsis* (ppi mutant) Designation	Proposed function	Mutant phenotype	Reference
Toc159	atToc159 (ppi2) At4g02510	Import receptor/motor protein	albino	(Bauer et al. 2002)
	atToc132 (toc132) At2g16640	Import receptor	none to pale-yellow green	(Ivanova et al. 2004; Kubis et al. 2004)
	atToc120 (toc120) At3g16620	Import receptor	none	(Ivanova et al. 2004; Kubis et al. 2004)
	atToc90 (ppi4) At5g20300	Import receptor	none	(Hiltbrunner et al. 2004; Kubis et al. 2004)
Toc34	atToc33 (ppi1) At1g02280	Import receptor	pale-green	(Jarvis et al. 1998)
	atToc34 (ppi3) At5g05000	Import receptor	none, reduced root length	(Gutensohn et al. 2000; Constan et al. 2004a)
Toc75	atToc75-III At3g46740	Translocation channel	embryo lethal	(Baldwin et al. 2005)
	atToc75-I At1g35860	no (pseudogene)	N/A	(Baldwin et al. 2005)
	atToc75-IV At4g09080	Translocation channel ?	abnormal etioplasts, de-etiolation defect	(Baldwin et al. 2005)
Toc64	atToc64-III At3g17970	Import co-receptor	none	(Qbadou et al. 2006)
	atToc64-I At1g08980	amidase 1 (AMI1) (no TPR domain)	N/A	(Pollmann et al. 2003; 2006)
	atToc64-V At5g09420	Mitochondrial receptor	N/A	(Chew et al. 2004)
Toc12	atToc12 ? At1g80920	dnaJ homolog	N/A	(Becker et al. 2004b; Becker 2005)
Tic110	atTic110 At1g06950	Translocation channel, chaperone recruitment	embryo lethal	(Inaba et al. 2005; Kovacheva et al. 2005)
Tic40	atTic40 At5g16620	co-chaperone	chlorotic	(Chou et al. 2003; Kovacheva et al. 2005)
Tic20	atTic20-I At1g04940	Translocation channel	seedling lethal, albino	(Chen et al. 2002; Teng et al. 2006)
	atTic20-IV At4g03320	N/A	N/A	(Jackson-Constan and Keegstra 2001a)
Tic62	atTic62 At3g18890	Redox-regulation	N/A	
Tic55	atTic55 At2g24820	Redox-regulation	N/A	

Pea	*Arabidopsis* (ppi mutant) Designation	Proposed function	Mutant phenotype	Reference
Tic32	atTic32 At4g23430	Redox, calcium regulation	embryo lethal	(Hormann et al. 2004)
Tic22	atTic22-IV At4g33350	Formation of contact sites	N/A	
	atTic22-III At3g23710	N/A	N/A	
Tic21	atTic21 (cia5) At2g15290	Translocation channel or PIC1 permease ?	albino, chlorotic, precursor accumulation	(Teng et al. 2006; Duy et al. 2007)
Hsp93	atHsp93-V (ClpC1) At5g50920	chaperone	Retarded growth chlorotic	(Sjogren et al. 2004; Constan et al. 2004b; Kovacheva et al. 2005)
	atHsp93-III (ClpC2) At3g48870	chaperone	not visible	(Constan et al. 2004b; Kovacheva et al. 2007)
SPP (CPE)	atCPE At5g42390	Transit peptide removal	(antisense) seedling-lethal	(Zhong et al. 2003)

Embryo lethality indicated that plastid protein import into plastids is absolutely required to establish essential, housekeeping biosynthetic pathways inside the organelle. Wild type, pale green and albino phenotypes suggest either accessory-functions or partial redundancy within small families of homologues. In the following, we discuss the phenotypes and expression patterns of the members of the Toc-GTPase family in detail as their analysis permitted the definition of their roles in protein import sub-pathways. These rely on components homologous and mechanisms similar to those of pea Toc159 and Toc34 while their substrates vary. For a complete summary of phenotypes of the known *Arabidopsis* chloroplast protein import components please refer to Table 1.

6.1.1 ppi2: the Toc159 knockout mutant

The *Arabidopsis* plastid protein import mutant 2 (*ppi2*), has a revealing albino phenotype due to a T-DNA insertion in the *atTOC159* gene (Bauer et al. 2000; Asano et al. 2004). The *ppi2* albino phenotype results in seedling lethality when plants are grown on soil. Similar to proplastids, *ppi2* plastids in cotyledons of soil-grown plants are undifferentiated and lack thylakoid membranes. Major photosynthetic genes such as RbcL, RbcS, and LhcII (CAB) are transcriptionally repressed indirectly due to the chloroplast biogenesis defect. However, it is noteworthy that *ppi2* plastids still import and accumulate the corresponding proteins in small amounts indicating the existence of Toc159 independent import pathways. Moreover, the expression and import of many proteins not involved in photosynthesis such as Toc75 and Tic110 (Bauer et al. 2000) and pE1α (Smith et al. 2004) is not

affected in *ppi2*. This led to the conclusion that atToc159 is the major import receptor for photosynthetic protein import and that the residual import of proteins into *ppi2* may be mediated by the remaining Toc159 homologues.

6.1.2 ppi4/toc132/toc120: the knockout mutants of the Toc159 homologues

The phenotypes of knockout mutants of the other Toc159 homologues and complementation experiments with these mutants support the hypothesis of functionally specialized import receptors (Hiltbrunner et al. 2001a, 2004; Ivanova et al. 2004; Kubis et al. 2004). The single *toc120* and *toc90* (*ppi4*) mutants have no visible phenotypes throughout development, *toc132* single mutant plants reveal no or a very slight pale phenotype in young seedlings to clear yellow-green and reticulate phenotype in mature plants depending on the ecotype used (Ivanova et al. 2004; Kubis et al. 2004).

Double knockout plants revealed the functional overlap of the receptors. *Toc120 toc132* double knockout plants were reported either to be embryo or seedling lethal, consistent with their role in the import of essential housekeeping genes and functional redundancy of Toc132 and Toc120 in this process. Correspondingly, overexpression of either of the two genes was sufficient to rescue the *toc120 toc132* mutant phenotype (Kubis et al. 2004). In contrast ectopic expression of atToc159 was not able to complement.

Crosses between *toc90* and other Toc159 homologue mutants did not result in any new visible phenotype expect for the combination of *toc90* (*ppi4*) with *ppi2* (Hiltbrunner et al. 2004). The *toc90* mutation aggravated the *ppi2* albino phenotype. *Ppi2 ppi4* did not accumulate detectable amounts of the photosynthetic protein CAB whilst the import of housekeeping genes was unaffected, indicating that Toc90 – when compared to Toc159 - may have an accessory function in import of photosynthetic proteins (Hiltbrunner et al. 2004).

6.1.3 ppi1/ppi3: the knockout mutants of Toc33 and Toc34

Single mutants of the two Toc34 homologues in *Arabidopsis* display relatively mild phenotypes (Jarvis et al. 1998; Constan et al. 2004a). The *ppi1* mutant carrying a T-DNA insertion in the atToc33 gene reveals a pale green phenotype most pronounced in young leaves (Jarvis et al. 1998). *Ppi1* chloroplasts of young leaves are small and have poorly developed thylakoids. Similar but weaker than in *ppi2* a downregulation of nuclear genes encoding photosynthetic chloroplast proteins was observed in *ppi1* plants (Kubis et al. 2003). Lack of atToc34 in the *ppi3* mutant does not cause any obvious phenotype in green aerial tissues but a slight reduction in root length was observed (Gutensohn et al. 2004; Constan et al. 2004a). The observations that overexpression of atToc34 complements *ppi1* and that the *ppi1 ppi3* double knockout is embryo lethal indicating that the two proteins functionally overlap to support an essential function. Genomics and proteomics data suggest that the two receptors while overlapping have preprotein-recognition specific-

ity, i.e. Toc33 for photosynthetic precursors and Toc34 for housekeeping proteins (Kubis et al. 2003).

6.2 Expression patterns of Toc GTPases

The expression pattern of the different Toc GTPases was analysed in several studies by RT-PCR (Bauer et al. 2000; Yu and Li 2001; Ivanova et al. 2004), RNA-blot (Gutensohn et al. 2000; Kubis et al. 2003, 2004), Affymetrix expression (Vojta et al. 2004) and Western blot analyses (Ivanova et al. 2004). Reasonably consistent results were obtained. Toc159 turned out to be the most abundant and most regulated of the four members of the Toc159 family (Bauer et al. 2000; Kubis et al. 2004; Vojta et al. 2004). It is highly expressed in rapidly growing photosynthetic tissue and downregulated in roots consistent with its proposed function as the major import receptor for photosynthetic precursors. The expression pattern of atToc33 parallels the one of atToc159 pointing to a concerted function of these two Toc GTPases. In contrast atToc90, atToc120, atToc132, and atToc34 show lower and much more uniform expression levels in different tissues and developmental stages than atToc159 or atToc33, indicative of their function in the transport of other and more constitutively expressed precursor proteins.

6.3 Biochemical evidence for functional specialization of chloroplast import receptors

The biochemical studies by (Ivanova et al. 2004) indicated that atToc120 and atToc132 indeed assemble into Toc complexes distinct from those containing Toc159. By sequential immunopurification it was demonstrated that atToc120/atToc132 preferentially assemble with atToc34, whereas atToc159 preferentially assembles with Toc33. Neither Toc120 nor Toc132 was found associated with Toc complexes containing Toc159.

Further evidence for the role of the Toc159 homologues as key determinants of import substrate specificity came from precursor binding studies using transit peptides of some selected photosynthetic and non-photosynthetic constitutively expressed precursor proteins. Toc159 specifically interacted with the transit peptides of two photosynthetic proteins (pSSU, pFd) and the transit peptides of three different non-photosynthetic plastid proteins (pE1α, pL11, pPORA) did not compete for this binding (Smith et al. 2004). The opposite was observed for Toc132 that selectively bound to the transit peptide of the constitutively expressed protein pE1α, but much less to that of a photosynthetic precursor (pSSU) (Ivanova et al. 2004).

6.4 Substrate specificity of Toc-GTPase sub-pathways

The genetic and biochemical studies provided evidence for the existence of structurally and functionally distinct translocons in the outer membrane of plastids as

well as the existence of at least two different classes of import substrates (Fig. 3). The two classes of import substrates have been operationally defined as photosynthetic (preferred substrates of Toc159 and 33) and housekeeping proteins (preferred substrates of Toc132, -120, and -34). The molecular basis for the discrimination of these substrates by the Toc-GTPase receptors is not known. It is generally assumed, however, that increased expression and accumulation of a protein in the *ppi1* mutant suggests import via a Toc33-independent import pathway whereas reduced expression and accumulation suggests a Toc33-dependent import pathway. Interestingly, precursors belonging to groups of either upregulated or downregulated showed differential clustering of hydroxylated amino acids in the transit sequences of precursor proteins (Vojta et al. 2004). Thus, the distribution of amino acid residues along in the transit sequences may explain how different receptors discern their favoured substrates.

This may also explain previous observations that transit peptides contain information for the preferential import into a certain plastid type (Wan et al. 1996; Yan et al. 2006). More information on precursor protein subsets that use a specific pathway and the determinants in the transit peptides for one specific pathway is needed to further substantiate this view.

7 Toc/Tic independent "alternative" import pathways into the chloroplast

N-terminal plastid transit peptides are not a general requirement for chloroplast targeting. For example most proteins, which are targeted to the outer envelope membrane of the chloroplast, do not contain a cleavable targeting sequence. Outer envelope proteins were assumed to insert spontaneously (Schleiff and Klosgen 2001; Hofmann and Theg 2005a). But there is evidence that the insertion of several outer membrane proteins like OEP14, *Physcomitrella* OEP64 (Toc64) or DGD1 depends on nucleotides and/or involves proteins at the chloroplast surface (Hofmann and Theg 2005b). The proteins involved and the mechanism of OEP targeting and insertion are largely unknown. But competition studies with a Toc/Tic import substrate hint at an involvement of Toc components in this process. In fact the insertion of OEP14 has been demonstrated to be mediated by Toc75 (Tu and Li 2000; Tu et al. 2004) and it is likely that the insertion of other OEPs depend on the Toc import channel as well.

7.1 Import depending on internal targeting sequences

A number of chloroplast proteins traversing the outer membrane without a cleavable N-terminal transit peptide such as ceQORH (chloroplast envelope quinone oxidoreductase homologue) or Tic32 (Miras et al. 2002; Nada and Soll 2004) have been identified. The targeting information of both proteins is contained in their respective mature sequences, targeting of ceQORH depends on an internal domain

of 40 residues (Miras et al. 2002), targeting of Tic32 on the most N-proximal amino acids (Nada and Soll 2004).

7.2 Substrate dependent import

Another plastid protein to be mentioned in the context of alternative import pathways is the protochlorophyllide oxidoreductase A (PORA), an essential enzyme in the light-dependent etioplast to chloroplast transition. Pre-PORA has a cleavable N-terminal transit-peptide, but it is still under dispute whether it is imported via a unique pathway (Reinbothe et al. 2004) or the general Toc/Tic-dependent import pathway (Aronsson et al. 2000, 2003a, 2003b). The import of PORA was reported to depend on its substrate protochlorophyllide (Pchlide) (Reinbothe et al. 1997, 2005; Kim and Apel 2004) and to involve the outer membrane proteins OEP16 and Toc33 (Reinbothe et al. 2004). Recent studies with *Arabidopsis* mutants deficient in OEP16 and Pchlide b indicate that neither OEP16 nor Pchlide b is essential for prePORA import (Philippar et al. 2007).

These examples as well as the detection of other nucleus-encoded proteins without canonical transit peptide sequences in chloroplast proteomes (Friso et al. 2004; Kleffmann et al. 2004) point to the existence of alternative import routes into plastids, independent of the Toc/Tic import pathway (Fig. 4). One such alternative transport route may involve the secretory pathway.

7.3 Protein import via the secretory pathway

Surprisingly at first sight, proteomic studies revealed the existence of many chloroplast proteins that do not have a predicted transit peptide but a predicted signal peptide (SP) promoting ER targeting instead (Friso et al. 2004; Kleffmann et al. 2004). At the same time, a possible import route for such proteins independent of the "general" Toc/Tic pathway was discovered (Chen et al. 2004; Villarejo et al. 2005; Nanjo et al. 2006; Radhamony and Theg 2006). First evidence for a transport of proteins into higher plant plastids via a signal peptide (SP) dependent pathway came from studies by (Chen et al. 2004), who found that the SP of a rice α-amylase (αAmy3) is necessary and sufficient for its dual targeting to the extracellular compartment and to plastids. Later, two other proteins exclusively located in the chloroplast were convincingly demonstrated to traffic from the ER-Golgi system to the chloroplast (Villarejo et al. 2005; Nanjo et al. 2006). Both proteins, *Arabidopsis thaliana* α-carbonic anhydrase (CAH1) and *Oriza sativa* NPP (nucleotide pyrophosphatase/phosphodiesterase) are N-glycosylated plastidal proteins and their chloroplast accumulation is inhibited by brefeldinA – a fungal antibiotic affecting Golgi-mediated vesicular transport. In contrast accumulation of a chloroplast protein taking the Toc/Tic import pathway was not brefeldinA-sensitive (Villarejo et al. 2005). The data from the proteomics studies and the

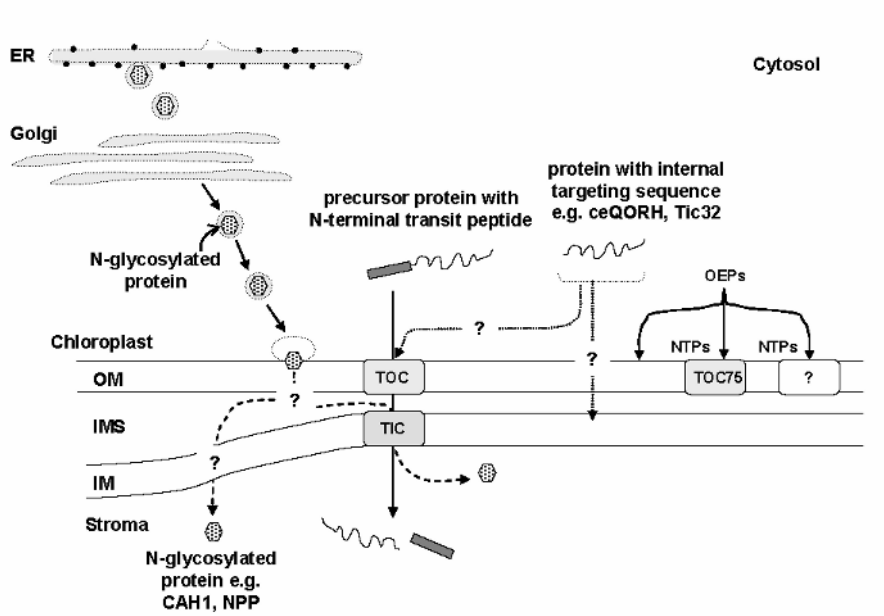


Fig. 4. Import pathways into the chloroplast (from left to right): Some stromal targeted proteins enter the endoplasmic reticulum and are transported via the Golgi apparatus to the chloroplast. During this passage they become glycosylated. They enter the chloroplast by the fusion of a Golgi-derived vesicle with the outer envelope membrane. The import routes of glycosylated proteins inside the chloroplast are not known but might involve Tic components. Precursor proteins with an N-terminal transit-peptide take the general import pathway via the Toc/Tic system. Chloroplast targeted proteins with internal targeting sequence like the inner envelope proteins ceQORH or Tic32 take a so far unknown import pathway most likely independent on the Toc/Tic system. Outer envelope proteins (OEPs) have been demonstrated to insert either spontaneously or dependent on energy (NTPs) and/or proteins in the outer membrane (e.g. Toc75).

immuno-detection of several N-glycosylated proteins in the chloroplast stroma indicates that a larger group of chloroplast proteins may be transported by a trafficking pathway involving the ER-Golgi system.

Although it is well established that the import into the so-called "complex" plastids of many algae and apicomplexan parasites occurs via the secretory pathway (Waller et al. 2000; Nassoury and Morse 2005), the involvement of SP and ER in plastid protein import of higher plants is an exciting new development in the field. Algal chloroplast protein precursors carry bipartite targeting signals consisting of the signal peptide and a stromal targeting domain (Sulli et al. 1999; Kilian and Kroth 2005) suggesting successive action of the secretory and the Toc/Tic pathways. In contrast the newly identified higher plant proteins predicted to use the secretory pathway only bear the signal peptide, suggesting a mechanism diverging from that in algae.

We predict that future research will unravel the components of glycosylated protein trafficking to the chloroplast including those involved in translocation across the envelopes. The mechanisms of the Toc-GTPases, regulation of import and the ATP-driven energetics are still far from being completely resolved and will remain major topics in the field in the next years.

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