

The TOC complex: Preprotein gateway to the chloroplast

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A B S T R A C T

Photosynthetic eukaryotes strongly depend on chloroplast metabolic pathways. Most if not all involve nuclear encoded proteins. These are synthesized as cytosolic preproteins with N-terminal, cleavable targeting sequences (transit peptide). Preproteins are imported by a major pathway composed of two proteins complexes: TOC and TIC (Translocon of the Outer and Inner membranes of the Chloroplasts, respectively). These selectively recognize the preproteins and facilitate their transport across the chloroplast envelope. The TOC core complex consists of three types of components, each belonging to a small family: Toc34, Toc75 and Toc159. Toc34 and Toc159 isoforms represent a subfamily of the GTPase superfamily. The members of the Toc34 and Toc159 subfamily act as GTP-dependent receptors at the chloroplast surface and distinct members of each occur in defined, substrate-specific TOC complexes. Toc75, a member of the Omp85 family, is conserved from prokaryotes and functions as the unique protein-conducting channel at the outer membrane. In this review we will describe the current state of knowledge regarding the composition and function of the TOC complex.

Keywords:
Protein targeting
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1. Introduction

One of the most important characteristics of photosynthetic eukaryotes is the presence of a family of interrelated organelles termed plastids. The plastids are tissue specific with specialized functions. All plastids are derived from a photosynthetic progenitor closely related to modern cyanobacteria. As the result of around 1.5 billion years of evolution, plastids became essential organelles harbouring some of the most important cellular processes of plants [1]. The chloroplast is the best characterized member of the plastid family, particularly because it is the home of photosynthesis. However, numerous other biosynthetic pathways including amino acid or lipid synthesis are also localized in chloroplasts. These pathways require numerous protein factors. Originally, most of these were encoded by the plastid progenitor. Over time, plastid genomes underwent a large reduction where most of the ancestral genes were transferred to the nucleus. Modern plastid genomes therefore encode only around 100 proteins. In contrast, the total proteome of *Arabidopsis thaliana* chloroplasts has been estimated to consist of between 2100 and 4500 proteins, the majority being encoded in the nuclear genome [2]. The products of these nuclear genes need to be imported into the plastid (Fig. 1).

Although some imported proteins may use other pathways, the vast majority engage the Translocons at the Outer and Inner membrane of the Chloroplasts (TOC and TIC complexes, respectively) (Fig. 1). These proteins are synthesized as preproteins with an amino

terminal targeting sequence known as the transit peptide. This peptide is removed upon arrival inside the chloroplast by the stromal processing peptidase (SPP) (Fig. 2). This review will focus on the TOC complex while the events at the TIC complex will be discussed elsewhere in this issue [3].

Elegant immunoelectron microscopy experiments demonstrated that the translocation process occurs at contact sites between the outer and inner envelope membranes [4]. Although, "holocomplexes" consisting of components of both the TOC and TIC complexes have been isolated, it is not known whether they physically constitute the contact sites.

Translocation across the chloroplast envelope has at least three biochemically distinguishable steps [4,5]. These steps were defined using the *Pisum sativum* (pea) chloroplast system:

- 1) The binding of the preproteins at the chloroplast surface which is a reversible and energy independent process [5,6].
- 2) The insertion across the outer membrane or the "early import intermediate" stage, which is irreversible and requires a low concentration of ATP ($\leq 100 \mu\text{M}$) [4]. It is inhibited by non-hydrolyzable analogs of GTP [5-9]. This stage has been dissected into three ATP-dependent phases (energy-dependent association, insertion and penetration), defined by the precursor state. These three steps differ by their requirement in temperature and energy [10].
- 3) Translocation simultaneously across both envelope membranes into the chloroplast stroma followed by the removal of the transit peptide ("late intermediate stage"). This stage requires millimolar concentrations of ATP ($> 1 \text{ mM}$) in the stroma [11,12].

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2. The TOC complex composition

The first attempts to isolate components of the TOC complex in the early 1990s resulted in the identification of three components of the outer and one at the inner envelope membranes of pea chloroplasts [4,13]. These were later confirmed in *Arabidopsis*. At the time they were termed either *Import intermediate Associated Proteins* or *Outer (or Inner) Envelope Proteins*, IAP/OEP34, IAP/OEP75, IAP/OEP86 [8,14–16] and IAP100/IEP110 [17]. They have been renamed using the TOC–TIC nomenclature as Toc34, Toc74, and Toc159 (for IAP/OEP86) and Tic110. It is important to note that several groups identified Toc159 as an 86 kDa protein. Only later was it recognized that the full length protein is much larger having a mass of 159 kDa. Thus, it appears that Toc159 is proteolytically sensitive and occurs in at least two different forms [18].

Toc34 and Toc159 are integral GTPases at the outer membrane. They share homology in their GTP-binding domains (G-domain). Both are largely exposed at the chloroplast surface and were therefore early on considered as candidates for primary receptors of cytosolic preproteins. The two GTPases function in conjunction with the β -barrel membrane channel Toc75 which is a member of the bacterial Omp85 family [19]. Together, these three proteins form the TOC core complex and appear to be sufficient for *in vitro* translocation of a precursor protein in lipid vesicles [20]. The mass of this core complex has been estimated at between 500 kDa and 1 MDa in pea [21–24], and around 1 MDa in *Arabidopsis* [23]. This wide range of masses suggests that at least one of the core proteins is present in

more than one copy. In pea, stoichiometries of 4–5:4:1 or 3:3:1 between Toc34, Toc75, and Toc159 have been determined [22,24]. However, the variations in the observed size and stoichiometries of the complex suggest that a clear stoichiometry may be difficult to establish. This may result from dynamic complex composition, the presence of different forms of Toc159 or even the experimental conditions; notably, Toc159 was present in the short 86 kDa form in the ~500 kDa complex [24]. Cryoelectron microscopy structural analyses suggest that the pea TOC core complex forms toroidal particles with a diameter of 13 nm and a height of 10–12 nm. The cryostructure revealed a central finger domain surrounded by a solid outer ring. The finger domain divides the toroidal cavity into four distinct pore-like domains; in agreement with the proposed stoichiometry of 4–5:4:1, each of the four pore-like substructures may consist of one molecule of Toc75 and one molecule of Toc34, and the unique Toc159 molecule would occupy the central position and be identical with the finger domain [24].

In addition to the TOC core components, Toc64 and Toc12 were later identified as new components of the complex. These two components are involved in the recognition of the preproteins and tethering of an intermembrane space Hsp70 to the exit site of the TOC complex, respectively [25,26].

The TOC complex has been shown to interact with cytosolic factors (Fig. 2), such as heat shock or regulatory proteins. These factors are implicated in the guiding of cytosolic preproteins to the chloroplast envelope. Two different pathways have been described so far. The first one is specific for preproteins with a phosphorylated transit peptide

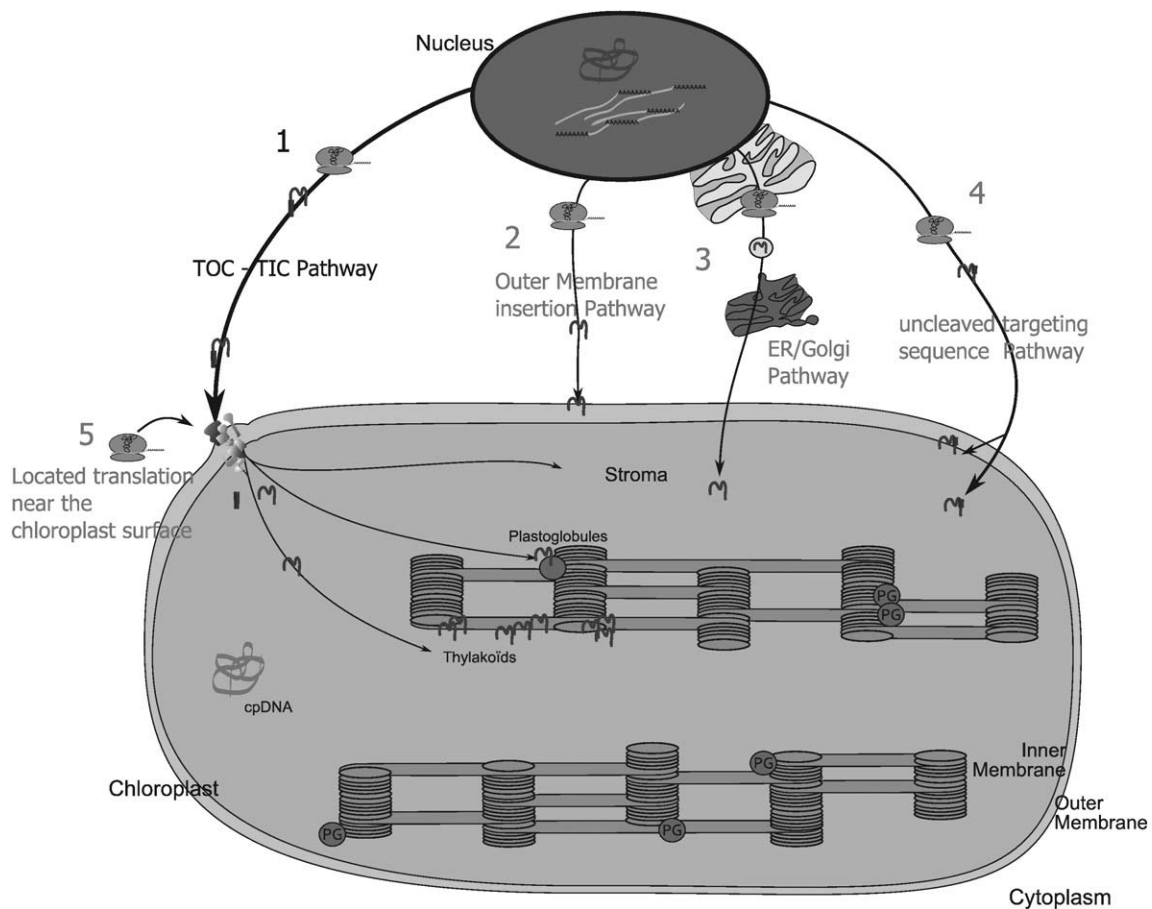


Fig. 1. An overview of the different pathways for the targeting of nuclear encoded chloroplastic proteins. 1) The TOC–TIC is the major route for proteins localized in the chloroplast, 2) most proteins localized in the outer membrane of the chloroplast reach their destination by direct insertion or receptor mediated pathways [116], 3) some proteins take an endoplasmic reticulum and Golgi pathway and may be glycosylated along the way [117], 4) an alternative pathway for proteins lacking recognizable transit peptides, or with non-cleavable, internal targeting sequences [118] and 5) in *Chlamydomonas reinhardtii* LHCII translation is located in the close vicinity of the chloroplast surface [103].

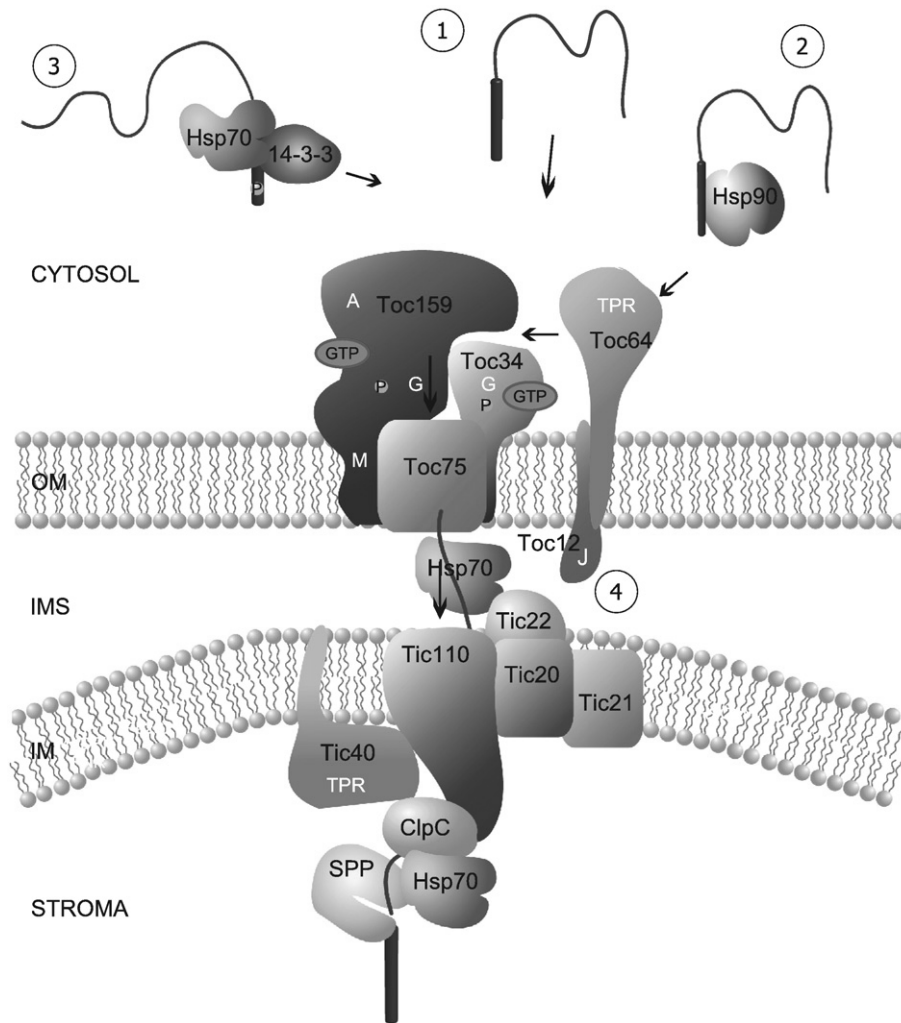


Fig. 2. The Translocons at the Outer (TOC) and (TIC) Inner envelope membranes of the chloroplast. The schematic illustration of the TOC complex indicates the targeting of preproteins to the TOC complex and their subsequent transfer to the TIC complex and the stroma. Preproteins may use three different ways to reach the Toc75 channel, 1) direct binding to the two TOC receptors Toc34 and Toc159; 2) some non-phosphorylated preproteins use the Toc64/Hsp90 pathway; 3) preproteins phosphorylated at their transit peptide use the “guidance complex” containing Hsp70 and 14-3-3. After outer membrane (OM) translocation, 4) Toc12, Hsp70 and Toc22 enable preprotein transfer across the intermembrane space (IMS). Finally, the preproteins cross the inner membrane (IM) via the TIC complex followed by transit peptide cleavage by the stromal processing peptidase (SPP). Only a minimal TIC complex without Redox and Ca^{2+} regulatory components is depicted.

and requires cytosolic Hsp70 and 14-3-3 proteins to form a guidance complex. The second pathway is used by non-phosphorylated preproteins. These bind to Hsp90 which requires Toc64 as co-receptor of Toc34 [10,27–29].

Since the completion of its genome in 2000 [30], *Arabidopsis* has become the favorite model system in plant cell biology because it provides a full arsenal of molecular genetic tools. The *Arabidopsis* genome revealed a surprising complexity of potential Toc proteins; the three core components are encoded by gene families rather than unique genes. Pea Toc34 has two paralogs in the *Arabidopsis* genome (atToc33 and atToc34), whereas four are found for each of Toc75 (Toc75-I, Toc75-III, Toc75-IV and Toc75-V/OEP80) and Toc159 (atToc159, –132, 120 and –90). It has been observed that distinct TOC core complexes exist in *Arabidopsis* as combinations of the different paralogs of Toc34 and Toc159 (Fig. 3) [31].

2.1. The receptors, Toc34 and Toc159

Translocation of the proteins across chloroplast envelope is an energetically costly process. At the early intermediate stage this energy appears to be supplied in part by GTP-hydrolysis as non-

hydrolyzable GTP analogs inhibit the process [15]. This GTP-dependence has been attributed to the members of the Toc34 and Toc159 families [32,33]. The members of each of the two families share a common domain organization. The Toc159 homologs have a C-terminal domain anchoring the proteins in the outer membrane (M-domain). The central GTP-binding domain (G-domain) and a highly acidic domain (A-domain) at the N-terminus face the cytosol. The recombinant A-domain of Toc159 is intrinsically disordered and may extend far into the cytosol [34]. The M-domains of Toc120, Toc132 and Toc159 are rather hydrophilic and do not exhibit typical hydrophobic transmembrane helices. The insensitivity to protease treatment in isolated chloroplasts is evidence to the membrane-protected nature of the M-domains [8,14,35]. The G-domain of Toc34 also faces the cytosol and is anchored in the outer membrane by a short hydrophobic sequence near the C-terminus [8].

Interestingly, the G-domains of Toc34 and Toc159 interact and this is believed to be an important element of the import mechanism. Both homodimerization and heterodimerization of the G-domains have been observed using biochemical methods [36–43]. Analysis of point mutations in *Arabidopsis* Toc33 (R130A and F67A) showed that abolishing receptor dimerization has no effect on the binding of

preproteins to the TOC complex, but is important for the initiation of the following translocation across the outer membrane [44]. Recently, experiments in *Arabidopsis* and *Nicotiana tabacum* (tobacco) protoplasts have shown that the G-domains of *Arabidopsis* Toc33 and Toc159 also form heterodimers *in vivo* [45]. GTP-binding proteins are known to act as “molecular switches” by alternating between active GTP-bound and inactive GDP-bound states [46]. Transition between the active and inactive states occurs by the hydrolysis of bound GTP, followed by the exchange of GDP by GTP. This usually involves factors known as GTPase Activating Proteins (GAP) or Guanine Exchange Factors (GEF) [47], but to date no specific factors have been identified for the TOC GTPases. However, it has been demonstrated that the presence of preproteins stimulated the hydrolysis rate of Toc34 [48,49] suggesting a GAP function of import substrates.

2.1.1. The Toc34 family

The Toc34 family has been named after pea Toc34 which was the first member of the family to be identified. Toc34 specifically coisolated with preproteins at the early and late import intermediate stages [5,8,14,16]. To date, in pea and algae, Toc34 is a unique protein, whereas in *Arabidopsis* and *Physcomitrella patens* (a moss), two and three genes are present, respectively. The crystal structures of pea Toc34 and *Arabidopsis* Toc33 in the either the GDP- or GMPPNP-bound state show homodimers involving the nucleotide binding sites on each of the monomers [50,51]. However, dimerization and GTPase activity may not be interdependent, as mutation of the arginine at position 130 of Toc33 abolishes dimer formation [41–43,50] without affecting GTPase activity by itself [41].

In *Arabidopsis*, null mutants of the Toc33 and Toc34 GTPases do not have strong phenotypes [52,53] (Table 1). The Toc33 mutant, *ppi1* (plastid protein import 1) showed a pale-green phenotype correlating with an abnormal ultrastructure of the chloroplast with smaller than normal and less developed thylakoids [53,54]. The *ppi1* mutant also exhibited a decrease in the expression of nuclear encoded chloroplast proteins, especially those involved in photosynthesis [55]. The Toc34 mutant, *ppi3* (plastid protein import 3), has no visible phenotype other than a decrease in root length [52]. However, the double null mutant *ppi1–ppi3* has an embryo-lethal phenotype [52]. Taken together these data show that Toc33 and Toc34 are partially overlapping which are

not redundant functions in *Arabidopsis*, especially with regard to their client import substrates (Fig. 3).

Pea Toc34 binds with a higher affinity to phosphorylated preproteins, and phosphorylation of Toc34 itself has been proposed to regulate its activity [56,57]. A corresponding kinase activity has been identified [56]. Despite the fact that *in vitro* phosphorylation of pea Toc34 inhibits both GTP and preprotein binding [48,49,57], modification of Toc33 phosphorylation site in *Arabidopsis* to mimic phosphoserine did not affect *in vivo* complementation of the Toc33 mutant, *ppi1* [58].

2.1.2. The Toc159 family

The first member of the Toc159 family was isolated from pea chloroplasts as an 86 kDa protein, hence the name Toc86 [5,8,14,16]. It later turned out that the 86 kDa fragment was a proteolytic fragment of a much larger protein and lacked the N-terminal A-domain [18,21]. Initial experiments showed that Toc159 was chemically crosslinked to the preprotein at the preprotein binding phase and that it was associated with the early import intermediate along with Toc34 and Toc75 [5,59]. These results suggested that Toc159 may be a primary receptor of preproteins. Interestingly, Toc159 binds only non-phosphorylated precursors [60].

A soluble form of Toc159 was reported in *Arabidopsis*, led to the proposition that it may bind preproteins in the cytosol and target them to the chloroplast envelope. Alternatively, the soluble form may simply represent an assembly intermediate [37]. However, the nature of the soluble form has been challenged and was attributed to low density membrane fragments occurring during fractionation of pea chloroplasts [60]. Currently, this issue remains unresolved.

Recently it has been proposed that the TOC complex interacts with actin and that the interaction is mediated by Toc159 [61]. The role of this interaction remains to be understood, but among the possible explanations is a role in gravitropism which involves actin [62]. Gravitropism has recently been linked to the TOC complex by the observation that Toc75-III and Toc132 are responsible for enhancing root gravitropism [63].

As mentioned before, three additional isoforms of Toc159 exist in *Arabidopsis* (Table 1): Toc90 (lacking an A-domain), Toc120 and Toc132 [55,56] (Fig. 3). The A-domains of Toc120 and Toc132 [64,65] diverge from each other and from that of Toc159 both in length and amino acid sequence. However, the net negative charge as well as

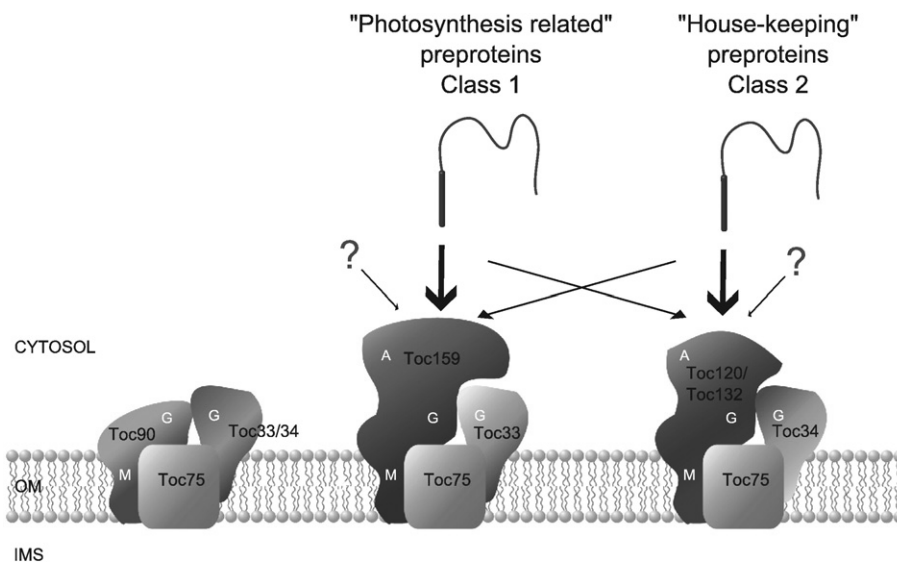


Fig. 3. In *Arabidopsis*, different classes of preproteins use distinct TOC complexes. TOC GTPases are functionally specialized, and exist in two different TOC complexes dedicated to the import of two different groups of preproteins. Toc33 and Toc159 associate in a TOC complex specific for photosynthesis-related preproteins (Class 1). This class comprises very abundant preproteins such as LHClI and the small subunit of Rubisco. Toc120 and Toc132 associate preferentially with Toc34 to form a TOC complex specific for non-photosynthetic or “house-keeping” preproteins (Class 2). A fourth homolog, Toc90, is present in *Arabidopsis*. It may be partially redundant with Toc159. To date it is not known whether Toc90 associates preferentially with either one of the two Toc34 homologs.

Table 1
Arabidopsis TOC proteins.

Components	<i>Arabidopsis</i> genes	AGI number	Proposed function	Protein characteristics	Mutant name	Null-mutant phenotype	References
Toc159	<i>TOC159</i>	At4g02510	Import receptor/motor function	GTPase, 3 domains structure	<i>ppi2</i>	Albino	[34,35]
	<i>TOC132</i>	At2g16640	Import receptor for house-keeping proteins		<i>toc132</i>	None to pale-yellow	[30,69]
	<i>TOC120</i>	At3g16620	Import receptor for house-keeping proteins		<i>toc120</i>	None	[30,69]
	<i>TOC90</i>	At5g20300			<i>ppi4</i>	None	[84]
Toc75	<i>TOC75-I</i>	At1g35860	Pseudo gene	POTRA domain, β -barrel structure	-	N/A	[83]
	<i>TOC75-III</i>	At3g46740	Major translocation channel		<i>toc75-III</i>	Embryo-lethal	[83]
	<i>TOC75-IV</i>	At4g09080			<i>toc75-IV</i>	Abnormal etioplast, de-etiolation defect	[83]
	<i>TOC75-V (atOEP80)</i>	At5g19620			<i>oep80</i>	Embryo-lethal	[78]
	<i>TOC64-I</i>	At1g08980	Amidase 1 (AMI1)		Amidase, 3 TPR domains	<i>toc64-I</i>	None
<i>TOC64-III</i>	At3g17970	Import co-receptor	<i>toc64-III</i>	Reduced import rate/none		[28,95]	
<i>TOC64-V</i>	At5g09420	Mitochondrial receptor	<i>toc64-V</i>	None		[95]	
Toc34	<i>TOC33</i>	At1g02280	Import receptor	GTPase	<i>ppi1</i>	Pale-green	[52]
	<i>TOC34</i>	At5g05000	Import receptor for house-keeping proteins		<i>ppi3</i>	None, reduced root length	[51]
Toc12	<i>TOC12</i>	At1g80920	Co-chaperone	J-domain	-	N/A	[25]

the preponderance of acidic amino acids is quite similar in the different A-domains [64].

Complementation studies of the Toc159 deletion mutant (*ppi2*, plastid protein import 2, see next paragraph) in *Arabidopsis* have revealed that the A-domain is dispensable. "Toc86" (or Toc159GM) consisting only of the G- and M-domains rescued the albino phenotype of the Toc159 deletion mutant [66,67]. Thus, these results indicate that the "Toc86" form of Toc159 most likely is an active protein. While clearly the A-domain is non-essential, it cannot be ruled out that it may be somehow functionally redundant with the A-domains of other Toc159 homologs [35].

The differences between the mutant phenotypes of the Toc159 family members suggested distinct receptor functions. *ppi2* exhibits an albino phenotype which is lethal when the mutant is grown on soil. At the molecular level, proteins involved in photosynthesis are particularly affected and fail to accumulate (unlike the so called "house-keeping" proteins which are constitutively expressed). This finding suggested a specific role for Toc159 in the import of photosynthetic proteins (Class 1 import substrates [68]) and indicated that other receptor systems are present to import the "house-keeping" proteins (Class 2 import substrates [68]). Interestingly, the photosynthesis-related nuclear genes encoding the photosynthetic proteins are downregulated at the transcriptional level [35]. Recently it has been proposed that, in *ppi2*, this downregulation of photosynthesis-related nuclear genes may be mediated by a new plastid-to-nucleus retrograde signaling pathways involving GUN1 [69].

Double mutants of Toc120 and Toc132 genes displayed either an albino [70] or an embryo-lethal phenotype [31], whereas the respective single mutants had no or only a weak phenotype [31,70]. The overexpression of either Toc120 or Toc132 (but not of Toc159) complemented the double mutant phenotype, indicating a significant functional overlap between the two proteins and a function distinct from that of Toc159 [70]. While *ppi2* specifically affected aerial tissues, the *toc132toc120* plants exhibit structural abnormalities in the roots suggesting a role in the import of house-keeping substrates or in non-photosynthetic tissues [70].

Analysis of *Arabidopsis* TOC complexes indicated that Toc120 and Toc132 proteins are present in complexes that excluded Toc159 [31], and are present in the protein import complex of proplastids [71] (Fig. 3). Moreover, Toc34 was present in Toc120/132-comprising complexes, whereas Toc33 was specific for those comprising Toc159 [31,72]. Taken together with the phenotype of the mutants, this finding supports the idea that a functional specialization occurs in *Arabidopsis* TOC GTPases, and that differential combination of TOC GTPases defines at least two distinct, substrate-specific pathways for preprotein import

(Fig. 3) [31,70]. It remains to be seen how preproteins are targeted to one or the other TOC complex, but recently specific sequence motifs have been identified in the Rubisco small subunit transit peptide (RbcS). Particularly, it has been shown that the serine residues in the N-terminal 12-amino acid segment of RbcS transit peptide were critical for the Toc159 dependent import of the preproteins [73].

2.2. The channel, Toc75

The channel in the outer membrane of chloroplast is formed by the Toc75 protein. *In vitro* analysis has demonstrated that Toc75 interacts with preproteins during import [5,59], and that it is associated with envelope-bound import intermediates [15]. Moreover, antibodies against Toc75 inhibit import [74].

Toc75 has a two-domain structure typical of the Omp85 family, an N-terminal domain containing POTRA signatures and a C-terminal β -barrel domain formed by β -strands [75]. The barrel domain of pea Toc75 is predicted to consist of 16 or 18 β -strands [76]. The Omp85 family comprises proteins essential for integration of β -barrel protein substrates in the outer membrane in Gram-negative bacteria and mitochondria (Sam50) [77], and this may also be the case for OEP80 in the chloroplast [78].

Patch clamp analysis of planar lipid bilayers has shown that reconstituted pea Toc75 forms a voltage-gated ion channel interacting specifically with precursor proteins [79]. The pore size has been estimated to be 14 Å at the narrowest part [80]. The N-terminal part of Toc75 is characterized by the presence of three POTRA domains, thought to assure secondary functions such as TOC complex assembly, preprotein recognition or chaperone-like activity [76].

Toc75 is the only protein of the TOC complex to possess an N-terminal cleavable targeting sequence; however, it is an unusual bipartite sequence. Its N-terminal part consists of a classical transit peptide, and this part reaches the chloroplast stroma where it is cleaved by the stromal processing peptidase. The C-terminal part of the bipartite targeting sequence spans the intermembrane space and is cleaved by an envelope-bound type-I signal peptidase [81].

A polyglycine stretch in the C-terminal part appears to play an essential role in retaining Toc75 at the outer chloroplast membrane. The role of the polyglycine stretch has not been elucidated for the moment, but it has been hypothesized that the stretch could prevent association of Hsp70 to the intermediate form of Toc75 (after cleavage of the transit peptide) before complete insertion, thereby assuring correct folding of Toc75 at the envelope [81,82].

To date two Toc75 coding genes are known to be present in the pea genome: Toc75, coding for the protein-conducting channel and Toc75-V

discovered on the basis of its sequence similarity with the bacterial homologue. It has been proposed that Toc75-V could be a direct ortholog of the cyanobacterial ancestor, and Toc75 a paralog [83].

In *Arabidopsis*, at least three Toc75 coding genes have been reported, Toc75-III, Toc75-IV, and Toc75-V and one pseudogene Toc75-I, and named according to their chromosome location [65] (Table 1).

Toc75-III, the closest homolog of pea Toc75, is most likely the channel of the *Arabidopsis* TOC complex. Toc75-III was present in an immunocomplex isolated using an affinity-purified antibody against Toc33 [37]. Furthermore, the Toc75-III deletion mutant is embryolethal, indicating an essential role in plastid development [84,85].

A second homolog, Toc75-IV having a shorter N-terminal region lacking the POTRA domain and five of the eighteen predicted β -strands [65,86], does not appear to be essential for protein import under normal growth conditions as the Toc75-IV mutant doesn't exhibit a visible phenotype. However, the ultrastructure of chloroplasts from dark-grown plants is altered in Toc75-IV mutants; suggesting a role in etioplasts [84].

A third homolog, formerly known as Toc75-V, has a molecular weight of 80 kDa and is thought to be involved in protein targeting to the chloroplast envelope. It has been named OEP80. It doesn't appear to be part of the TOC complex, and may be required for insertion of beta-barrel proteins in the outer membrane [87]. Comparative analysis of chloroplast import of OEP80 and Toc75 has led to the proposition of a second hypothesis regarding the origin of these genes; OEP80 and Toc75 ancestors may have diverged prior to the endosymbiotic event, and developed different roles in protein targeting mechanisms [87].

Toc75-I, the fourth homolog, has not gotten much attention as it is a pseudogene interrupted by a gypsy transposon insertion [84].

In silico analysis has shown that Toc75 is the only component of the TOC complex of cyanobacterial origin as it is derived from the secretory pathway of the original endosymbiont [88]. Toc33 and Toc159 appear to have been added to the core complex later on, but still at an early step of evolution as the components are common to all higher plants, green algae as well as mosses [88–90]. Existence of true TOC GTPase homologs in the red algae lineage is still in question, though putative homologs have been identified [89,91].

2.3. Other TOC proteins

2.3.1. Toc12

Toc12, a protein containing a J-domain, is located at the intermembrane face of the outer envelope [25]. Toc12 has been shown to interact with Toc64 [25]. *In vitro*, Toc12 interacts with Hsp70 proteins and enhances ATP hydrolysis by DnaK. A role for Toc12 may be to retain Hsp70 proteins in the close vicinity of the TOC complex, in order to bind to incoming preproteins and prevent them from slipping back into the cytosol at the “early intermediate stage”. A disulfide bridge in the loop region of the J-domain may be implicated in the redox regulation of the Toc12/Hsp70 interaction [25].

2.3.2. Role of Toc12 at TOC–TIC contact sites

Translocation across the dual membrane envelope is accomplished in a synergistic fashion, in which the TOC and TIC complexes are in immediate vicinity at the contact sites between the two membranes [4,92]. These contact sites may be mediated by proteins. To date, Toc12 and Tic22, both largely facing the intermembrane space, are strong candidates for “bridging” the two translocon complexes [25,59,93]. A model involving these two proteins as well as Toc64 and an Hsp70 protein has been proposed to explain the translocation across the intermembrane space [27].

2.3.3. Toc64

Toc64 is composed of three different functional regions: the N-terminal region contains a transmembrane region, the central domain

has homology with amidases and the C-terminal domain contains three tetratricopeptide motifs (TPR) [26]. Toc64 is loosely associated with the TOC core complex [26,28]. Toc64 has been proposed to be involved in the targeting of cytosolic preproteins to the TOC complex, the TPR domain acting as a docking station for the Hsp90 chaperone carrying preproteins [94]. Pea has one known gene for Toc64, whereas in *Arabidopsis* three paralogs have been found Toc64-I, Toc64-III, and Toc64-V, [65]. Toc64-III is targeted to the outer envelope membrane of chloroplasts [95], whereas the two others are not chloroplastic. Toc64-I, or AMI1, is a cytosolic protein reduced to the amidase region and lacking the transmembrane region as well as the TPR motifs. Toc64-I is non-essential for chloroplast protein import [96–98]. Toc64-V is a mitochondrial protein and has been proposed to be the plant analog of the animal and fungal component of the translocase of the outer membrane of mitochondria, TOM70 [97].

Mutant analysis has shown that chloroplast Toc64 is non-essential for chloroplast protein import both in *P. patens* and *Arabidopsis* [96,99]. Slight phenotypes were observed, such as modification of the chloroplast shape in moss [100], but even the *Arabidopsis* triple mutant does not exhibit any import phenotype [96]. The lack of an apparent function in import in these studies has put its proposed role in question and led to the suggestion to rename Toc64 as OEP64 [100].

3. Translocation process

3.1. Targeting of preproteins to the TOC complex

Chloroplast protein import is considered a post-translational process involving cytosolic factors to guide newly synthesized preproteins from the ribosomes to the chloroplast surface [37,86,101,102]. Recently, however, it has been demonstrated in *Chlamydomonas reinhardtii*, that LHCI proteins are translated in close proximity of the chloroplast surface which may provide an alternative to cytosolic guidance complexes [103]. In addition, direct interaction of cytosolic preproteins with the Toc complex and even the galactolipids in the envelope has been proposed as an initial recognition step.

The targeting of preproteins to the chloroplast envelope and subsequent translocation using the TOC–TIC pathway depends on the N-terminal transit peptide [104,105]. The transit peptide does not appear to acquire a specific secondary structure and has been suggested to form a “perfect random coil” allowing interaction with different classes of chaperones as well as insertion into the translocation channel [95].

The “guidance complex” is a heterooligomeric complex consisting of cytosolic Hsp70, a 14-3-3 protein and the preprotein (Fig. 2). In order to be recognized by 14-3-3 proteins in the guidance complex, preproteins have to be phosphorylated within their transit peptide enabling their subsequent delivery to the TOC complex and subsequent translocation [106].

A second targeting mechanism has been proposed for non-phosphorylated preproteins. These may bind to Hsp90 in the cytosol. Toc64 functions as a co-receptor for Hsp90–preprotein complex and in turn interacts with Toc34 to deliver the preprotein to the Toc75 channel [24,26,28] (Fig. 2).

In contrast, a number of studies have demonstrated that neither of the two cytosolic targeting systems are essential suggesting that additional systems (such as the localized translation of LHCI) exist [96,100,107].

Galactolipids at the chloroplast envelope (monogalactosyldiacylglycerol, MGDG or digalactosyldiacylglycerol, DGDG) play a role in protein import into chloroplasts. The relative roles of the two galactolipids in protein import remain unclear. *In vitro* experimentation showed an important function for MGDG, but this was not confirmed by *in vivo* analyses of a weak mutant (*mgd1*) [100,108–110]. On the other hand, a mutant lacking DGDG exhibited a severe defect in protein import [111,112]. Moreover, DGDG has been found in association with the TOC complex [24], suggesting that it may play

the more important role of the two. While a direct interaction of the transit peptide with the galactolipid containing lipid bilayer has been observed, it cannot be excluded that galactolipids also act indirectly on protein import through specific lipid requirements of TOC proteins.

Clearly many questions surrounding the targeting of preproteins to the chloroplast surface remain open and offer much potential for future research.

3.2. Outer membrane translocation

Two models currently exist to explain the translocation mechanism of preproteins across the chloroplast envelope, the “targeting model” and the “motor model”. They differ by the identity of the primary acceptor of preproteins, Toc34 and Toc159, and by the role of GTP-hydrolysis (Fig. 2).

In the “targeting model”, Toc159 is considered the primary preprotein receptor [37,72]. It is supported by evidence that Toc159 together with Toc75 is the major TOC protein interacting with preproteins in chemical cross-linking experiments carried out at the preprotein binding and early intermediate stage [5,6,59]. Toc159, together with the preprotein, enters the TOC core complex via heterodimerization with Toc34. The GTPase activity of Toc159 is thought to play a role in the integration of Toc159 into the outer membrane and the TOC complex, as it has been shown that a triple point mutation in the GTP-binding motif G1 failed to complement *ppi2* phenotype and led to accumulation of a soluble form of Toc159 [36]. Moreover, the recombinant G-domain of the *Arabidopsis* Toc34 homolog atToc33 inhibited insertion of synthetic Toc159 into the outer membrane [40–43,51]. The targeting model has been described as a pathway remotely analogous to the signal recognition particle (SRP) system of the endoplasmic reticulum which also contains two homologous GTPases [113]. In this view Toc159 and Toc34 may be seen as analogs of SRP and SRP-receptors respectively [15,25].

In the “motor model”, Toc34 is thought to function as the primary preprotein receptor. This model is principally based on the *in vitro* analysis of the TOC core complex in reconstituted proteoliposomes [20]. Toc34, in this model, is permanently associated with the TOC core complex and does not depend on GTP for its assembly (as has been proposed for Toc159 in the “targeting model”). Preprotein recognition by Toc34 depends on GTP-binding [20]. Upon GTP-hydrolysis, the preprotein is handed on to Toc159 which serves to drive translocation using one or more GTPase cycles, hence the term “motor model” [20,60]. Phosphorylated preproteins would have to be dephosphorylated before their transfer from Toc34 to Toc159 in the motor model.

Both models have been put somewhat in perspective by recent *in vivo* experiments examining the effects of Toc159 GTPase mutants A864R (binding but not hydrolyzing GTP) [114] and K868R (neither binding nor hydrolyzing GTP) [67]. Neither of the mutations resulted in a visible phenotype when transformed in *ppi2* plants. However, A864R chloroplasts were more efficient in *in vitro* protein import experiments than control chloroplasts whereas for K868R chloroplasts import efficiency was reduced. From these experiments it thus appears that the Toc159 GTPase is comparable to a switch which in its GTP-bound form tends to be in the “ON” state and “OFF” in its empty state. Interestingly, it was also demonstrated that non-hydrolyzable GTP analogs still strongly inhibited residual protein import in chloroplasts from either genetic backgrounds [9,10]. This suggests that GTPases other than Toc159 actively participate in the import process. Likely candidates are the Toc34 homologs or the other members of the Toc159 family.

4. Conclusions

Since the first evidence that preproteins bind to receptors at the surface of chloroplast [115], much progress have been made in the

understanding of the translocation across the outer chloroplast membrane. A multiprotein TOC complex has been described and presumably the three most important components have been identified and form at least two different types of TOC core complexes. The functional roles of the core components have been defined. Yet, the details of the GTPase mechanisms and the nature of regulatory factors at the core complex despite great efforts remain elusive. An important issue is the targeting of cytosolic preproteins to the TOC complex. An important issue is the targeting of cytosolic preproteins to the TOC complex. The recent description of localized translation of the LHClI preprotein near the surface of the chloroplasts suggests that preprotein targeting may in part be regulated at the mRNA level. These are exciting prospects for the research at the TOC complex in the next decade.

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