PROTEIN IMPORT INTO CHLOROPLASTS

Jürgen Soll and Enrico Schleiff

Chloroplasts are organelles of endosymbiotic origin, and they transferred most of their genetic information to the host nucleus during this process. They therefore have to import more than 95% of their protein complement post-translationally from the cytosol. *In vivo* results from the model plant *Arabidopsis thaliana* — together with biochemical, biophysical and structural data from other plants — now allow us to outline the mechanistic details of the molecular machines that facilitate this translocation. It has become clear that chloroplasts evolved a unique translocation system, which is inherited, in part, from their bacterial ancestors.

PLANT CELL BIOLOGY

PLASTID
A plant-specific family of organelles, the differentiation of which is dependent on the plant organ and on plant development.

PLASTIDS — such as chloroplasts, which carry out photosynthesis in the green parts of plants — are present in every plant cell. Chloroplasts originated from an endosymbiotic event, in which an ancestral photosynthetic cyanobacterium was taken up by a heterotrophic host cell that already contained mitochondria[1,2]. This endosymbiotic process led to a massive transfer of genetic information from the endosymbiont to the emerging host nucleus. A prerequisite for the successful completion of this process was the development and establishment of protein-import machinery for the import of chloroplast-localized, but now cytosolically synthesized, polypeptides. As chloroplasts are the most recent organelle to be added to the eukaryotic cell, several post-translational protein-targeting systems probably already existed in the host cell, for example, those belonging to mitochondria[3], peroxisomes[4] and the plasma membrane[5]. Therefore, the arising chloroplast protein-import system had to develop unique features to ensure organelle specificity, as the correct sorting of proteins in a eukaryotic cell is essential for its functionality.

The chloroplast proteome is estimated to consist of 3,500–4,000 polypeptides, whereas the coding capacity of the chloroplast genome rarely exceeds 200 genes[6,7]. Newly imported polypeptides are frequently integrated into protein complexes that also contain proteins that are synthesized inside the organelle. The most prominent examples are the two photosystems, the ATP synthase and the CO_2-fixing enzyme ribulose-1,5-biphosphate carboxylase oxygenase (Rubisco). Because of this dual genetic origin, there must be a tight coordination of transcription, translation and protein import between the parent cell and the organelle. Only the tight coordination of these processes in time, space and quantity ensures the successful biogenesis of the organelle.

Chloroplasts are highly structured and contain three distinct membrane systems — the outer- and inner-envelope membranes, which surround the organelle, and the thylakoid membrane network, which contains the photosynthetically active protein complexes (BOX 1). In addition, three soluble subcompartments — the space between the envelope membranes, the stroma and the thylakoid lumen — can be clearly distinguished. So, in addition to the targeting and import systems that are the main focus of this article, several intra-organelle sorting and transport systems must also be present in chloroplasts[8–10].

Although chloroplasts are thought to originate from a unique primary endosymbiotic event, many algae contain photosynthetically active chloroplasts that are surrounded by three or four membranes. These chloroplasts are called complex plastids and they originated from a secondary endosymbiotic event in which a photosynthetic eukaryotic cell was taken up by a non-photosynthetic eukaryotic host

Department für Biologie I, Botanik, Ludwig-Maximilians-Universität München, Menzingerstraße 67, D-80638 München, Germany.
Correspondence to J.S.
e-mail: soll@uni-muenchen.de
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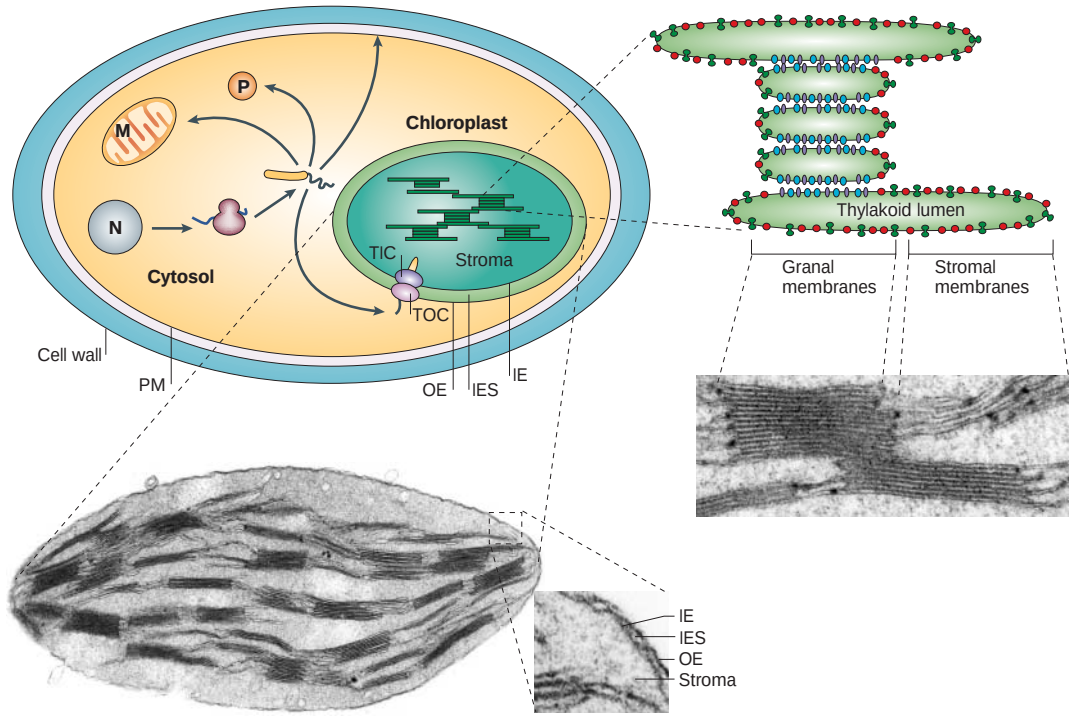
Box 1 | General outline of protein import and chloroplast ultrastructure



Polypeptides that are encoded in the nucleus (N) are translated in the cytosol and are post-translationally targeted by various targeting signals (depicted here in yellow) to several cellular compartments — for example, mitochondria (M), peroxisomes (P), plasma membrane (PM) and chloroplasts (see figure). Protein import into chloroplasts is achieved by two translocons called TOC and TIC that reside in the outer and inner envelope (OE and IE), respectively. The chloroplast is highly structured. It is composed of three membrane systems — that is, the OE and IE, and the thylakoid membrane network that contains the protein complexes that are involved in photosynthesis. In addition, three soluble spaces can be distinguished — that is, the inter-envelope space (IES), the stroma and the thylakoid lumen. Several other targeting and translocation systems are therefore also present in chloroplasts to direct proteins to each of the six subcompartments. An electron micrograph of an isolated *Pisum sativum* chloroplast is shown, and the enlargement on the far right shows the typical organization of thylakoids. Granal membranes are preferentially enriched in photosystem II (blue) and the cytochrome b_6f complex (purple), whereas stromal membranes are enriched in photosystem I (red) and the ATP synthase (green).

cell. Relic non-photosynthetic complex plastids called APICOPLASTS are also present in several intracellular parasites such as *Plasmodium falciparum* and *Toxoplasma gondii*, which are the causative agents of malaria and toxoplasmosis, respectively. Protein targeting and translocation into plastids is therefore of broad interest[11].

A general outline of chloroplast protein import

The vast majority of chloroplast proteins are synthesized as precursor proteins (preproteins) in the cytosol and are imported post-translationally into the organelle. Most proteins that are destined for the thylakoid membrane, the stroma and the inner envelope are synthesized with an amino-terminal extension called a presequence, or transit sequence, which is proteolytically removed after import (FIG. 1). The transit sequence is both necessary and sufficient for organelle recognition and translocation initiation. Preproteins that contain a cleavable transit peptide are recognized in a GTP-regulated manner[12] by receptors of the outer-envelope translocon, which is called the TOC complex[13]. The preproteins cross the outer envelope through an aqueous pore and are then transferred to the translocon in the inner envelope, which is called the TIC complex. The TOC and TIC translocons function together during the translocation process (FIG. 2). Completion of import requires energy, which probably comes from the ATP-dependent functioning of molecular chaperones in the stroma[14]. The stromal processing peptidase then cleaves the transit sequence to produce the mature form of the protein, which can fold into its native form.

From translation to the organelle surface

When the nascent polypeptide chain emerges from the ribosome it encounters a highly concentrated proteinaceous environment. Non-productive interactions or the exposure of hydrophobic amino-acid stretches result in aggregation and the loss of newly synthesized proteins. Molecular chaperones of the heat shock protein (HSP)70 FAMILY interact with non-native preproteins and keep them in a soluble, conformation that is not

APICOPLAST
A non-photosynthetic plastid that is surrounded by four membranes and that still synthesizes essential metabolites for the host.

HSP70 FAMILY
Heat shock proteins that have a molecular weight of 70 kDa and that function as molecular chaperones.

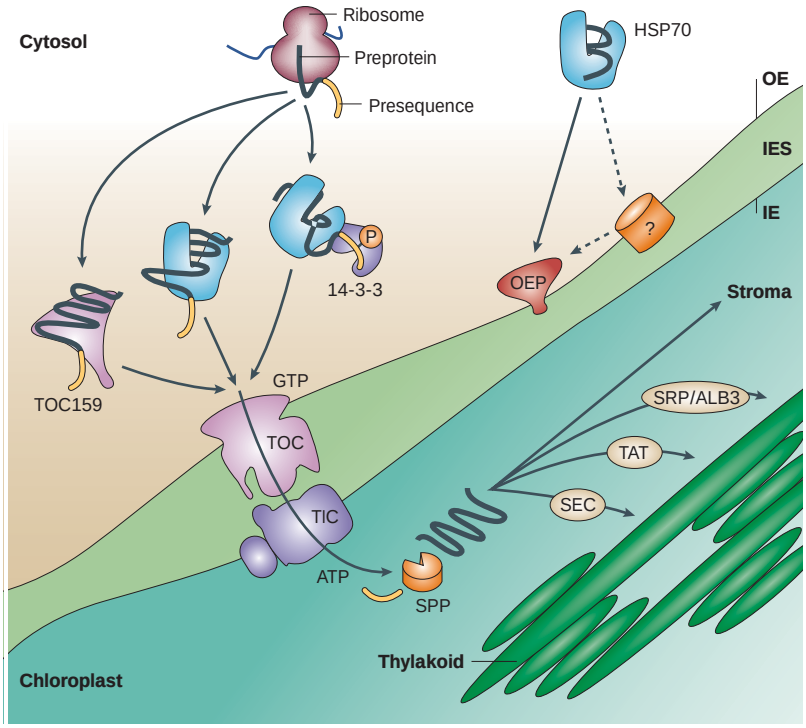


Figure 1 | **Protein-import pathways into chloroplasts.** In the cytosol, preproteins with an amino-terminal presequence or transit sequence (yellow) can form a cytosolic guidance complex on phosphorylation in the cytosol. This guidance complex consists of an HSP70 (heat shock protein-70) chaperone and a 14-3-3 dimer. Altenatively, non-phosphorylated preproteins can associate with TOC159 (translocon of the outer chloroplast envelope-159) or with HSP70. All these complexes bind to TOC receptors in a GTP-dependent manner. A soluble TOC159 receptor might shuttle between the cytosol and the TOC complex bringing preproteins to the organelle surface. In a joint effort between TOC and TIC, preproteins are imported across both membranes in an NTP-dependent manner. The transit sequences are cleaved off by the stromal processing peptidase (SPP). Mature proteins either fold and assemble in the stroma or are directed to the thylakoids by various pathways. Preproteins without a cleavable transit sequence are mostly bound by HSP70 and targeted to the outer envelope. They can insert spontaneously into the membrane *in vitro*, although the process might be facilitated by proteinaceous factors *in vivo*, which still need to be identified (highlighted by a question mark). ALB3, albino3; IE, inner envelope; IES, inter-envelope space; OE, outer envelope; OEP, outer-envelope protein; SEC, secretory pathway; SRP; signal-recognition particle; TAT, twin-arginine translocase; TIC, translocon of the inner chloroplast envelope.

fully folded[15–18]. This is also necessary because polypeptides have to cross the envelope translocons in a largely unstructured, extended conformation.

A common motif for targeting has not yet been identified in chloroplast transit sequences, but, in general, the transit sequences vary in length from 20–150 amino acids. Proteins that are localized in the thylakoid lumen contain bipartite targeting signals. The amino-proximal portion functions as a chloroplast-targeting, envelope-transfer domain, whereas the carboxy-proximal portion functions as a thylakoid-transfer domain[19–22]. Other preproteins are synthesized without a cleavable transit sequence and contain their targeting information in the mature part of the protein (FIG. 1). This group of preproteins includes almost all of the outer-envelope proteins[23], as well as one protein from the inner-envelope membrane so far[24]. Cleavable transit peptides carry an overall positive charge and are enriched in the hydroxylated amino acids serine and threonine. In aqueous solution, the transit peptide forms a random-coil structure[25]. Several, but not all, chloroplast preproteins can be phosphorylated in the transit sequence by a cytosolic ATP-dependent protein kinase[26]. Phosphorylation leads to the binding of 14-3-3 PROTEINS, which, together with HSP70, can form a cytosolic guidance complex[27] (FIG. 1). Preproteins that are bound to the guidance complex are imported into chloroplasts more quickly than monomeric preproteins. Phosphorylation in the cytosol might therefore select a subclass of preproteins for preferential import[26,27]. Phosphorylation is not directly involved in targeting, because mutating the phosphorylation site does not result in mistargeting *in vitro*. Furthermore, the nature of the kinase that performs this phosphorylation is still unknown, so *in vivo* evidence for the importance of precursor phosphorylation is still missing. Transit-sequence recognition is achieved by the receptor polypeptide of the TOC complex. Less is known about the mechanism of presequence-independent insertion into the outer envelope.

14-3-3 PROTEINS
A family of ubiquitous regulatory molecules that function through protein–protein interactions.

The TOC translocon

The subunits of the TOC translocon were initially identified by chemically crosslinking precursor proteins to neighbouring polypeptides or by membrane-complex isolation in the presence of detergent[28–33]. Due to the different energy requirements for preprotein binding (<50 μM NTP) and translocation (>100 μM NTP), different translocon subunits could be identified. TOC75 and TOC159 were typically found crosslinked to preproteins at low GTP and ATP concentrations[31,34], whereas TOC34 was mainly found to be crosslinked in the absence of GTP or ATP (REF. 35). From these studies, it was proposed that TOC159 functions as an initial receptor, whereas TOC34 has a later regulatory function. However, it should be noted that the experimental set up that was used was, biochemically, extremely complex. Chloroplasts always contain residual NTPs and NDPs, which, owing to the presence of myokinase and nucleoside diphosphate kinase[36], can result in the production of every type of NTP or interconversion between NTPs during the experiment. Nucleotide 'free' experiments might therefore actually not be completely nucleotide free. Furthermore, crosslinking experiments require that labelled preproteins accumulate at a certain site, so that they become detectable. Rapid kinetic intermediates are therefore difficult to identify in this type of experiment, so it is hard to deduce a clear series of steps in the import process from *in organello* studies.

Nevertheless combining the techniques that are described above resulted in the identification of four TOC translocon subunits — the two GTP-binding proteins TOC159 and TOC34 (REFS 28–30), the protein-import channel TOC75 (REFS 31,32,37), and TOC64 (REF. 38; FIG. 2). The former three subunits form a stable core complex of ~550 kDa, which has a stoichiometric ratio of 4:4:1 for TOC34:TOC75:TOC159 (REF. 39). In

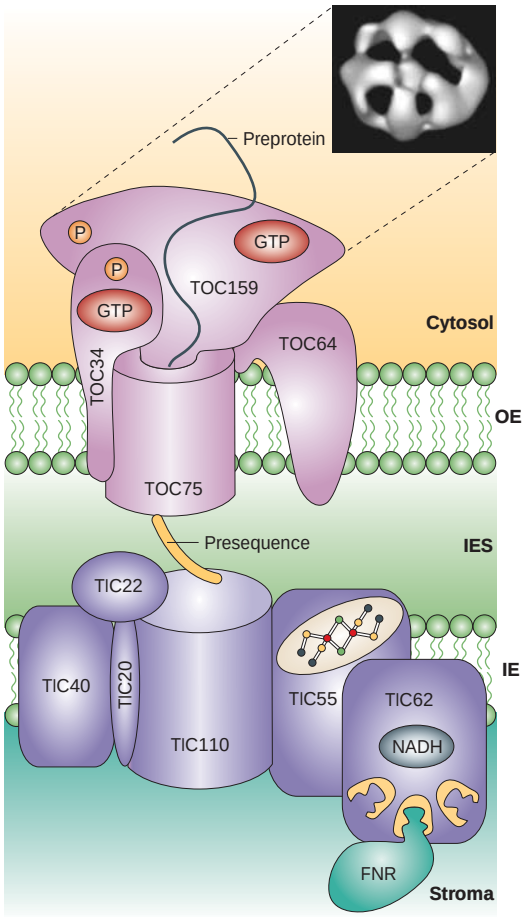


Figure 2 | **The chloroplast translocon complexes.** The import machines in the outer (TOC) and inner (TIC) envelope from *Pisum sativum* have distinct subunit compositions. The TOC core complex is formed by the GTP-dependent receptor TOC34, the import channel TOC75 and the GTP-driven motor protein TOC159. TOC34 and TOC159 can be regulated by protein phosphorylation. The insert shows a three-dimensional reconstruction of a negatively stained electron-microscopy image that was obtained of the purified TOC core complex from *P. sativum*. TOC64 might function as a docking protein for HSP70 (heat shock protein-70)-guided preproteins — it is only loosely attached to the core complex. TIC110 might form the translocation channel of the TIC complex, and TIC20 might also be part of this channel (please refer to the text for further details). TIC40, which is closely associated with TIC110, might function as a chaperone-recruiting site. TIC22, which is localized in the inter-envelope space, might coordinate TIC and TOC function. The redox components TIC55 and TIC62 are proposed to regulate translocation through the TIC complex, and the binding of ferredoxin NAD(P) reductase (FNR) to TIC62 allows the TIC complex to sense the redox state of the chloroplast. TIC62 also contains a conserved NAD(P)-binding site. TIC55 contains an iron–sulphur centre and a mononuclear iron-binding site. The figure reflects only the subunit composition of the TIC and TOC complex as published to date, and does not reflect stoichiometric ratios, definitive functions or correct protein topologies. The electron-microscopy insert in the figure was reproduced with permission from REF. 39 © (2003) The Rockefeller University Press.

T-DNA
(transfer DNA). The part of the *Agrobacterium tumefaciens* tumour-inducing (Ti) plasmid that is incorporated into the genome of infected plant cells. These conjugative plasmids can be used as tools to insert foreign DNA into plant cells.

PHOTOAUTOTROPHIC
Organisms — for example, plants or cyanobacteria — that use light as an energy source to convert inorganic material into organic matter.

SEED ABORTION
The development of a seed — that is, an embryo — is stopped because of a fatal error in differentiation.

the model plant *Arabidopsis thaliana*, several genes exist for every TOC subunit[40] (see below). Transcriptional control and organ-specific expression might result in the formation of TOC complexes that have different selectivities and translocation properties. Most of the *in vitro* biochemical data summarized below were obtained using TOC proteins from *Pisum sativum*, whereas the *in vivo* data were generally obtained from studies of *A. thaliana*. To distinguish between *P. sativium* and *A. thaliana* proteins, the prefixes *Ps* and *At* will be used.

TOC33/34. TOC34 is anchored by its carboxy-terminal tail, and most of the protein, including the GTP-binding domain, is exposed to the cytosol[30]. Reconstituted TOC34 can directly interact with preprotein[12,41]. Preproteins are recognized with a high affinity when TOC34 is in its GTP-bound form[12,42]. The low endogenous GTPase activity of TOC34 (REF. 30) is stimulated up to 50-fold by the preprotein[43]. Therefore, the preprotein seems to function as a GTPase-activating protein, and such proteins are key for the regulation of GTPases in various systems[44]. TOC34–GDP has a much lower affinity for the preprotein[42], which is subsequently handed to the next TOC subunit.

The next step in the TOC34 cycle is the release of GDP, which would normally be promoted by a GDP-exchange factor[45]. Such a factor has not yet been identified, but a domain of TOC159 could possibly fulfil this role (see below). The nucleotide-free form of TOC34 can then take one of two routes. First, it can be recharged with GTP and enter a new round of precursor recognition and binding or, second, it can be phosphorylated by an outer-envelope-localized, ATP-dependent protein kinase[46]. Phosphorylation inhibits GTP binding and therefore results in a complete switching off of the receptor. In *A. thaliana*, two homologues of *Ps* TOC34 are present[40] — *At* TOC33, which seems to be a functional analogue of *Ps* TOC34, can also be switched off by phosphorylation, whereas *At* TOC34 seems not to be phosphorylated[47]. *At* TOC34 might therefore function as a constitutive receptor for protein import into different plastid types (see below)[48,49] (FIG. 3).

A. thaliana has become a model organism, and it can be used for forward and reverse genetic approaches. The roles of several TOC and TIC subunits in chloroplast biogenesis have therefore been studied in plants containing either antisense-RNA constructs — which lead to a reduction, but not a complete absence, of the target protein — or T-DNA insertions, which lead to a complete knockout of the target gene product. The reduction or knockout of translocon subunits that are essential for translocon function should result in severe defects in chloroplast/plastid biogenesis (for example, a loss of the ability to grow PHOTOAUTOTROPHICALLY), or should lead to lethality, which is normally manifested as an embryo lethality that results in early SEED ABORTION.

The plastid-protein-import (*ppi*) mutants have been numbered consecutively and the first to be isolated was a knockout of *At toc33* (REF. 50; *At* TOC33 is a functional analogue of *Ps* TOC34 (see above)). This *ppi1* mutant

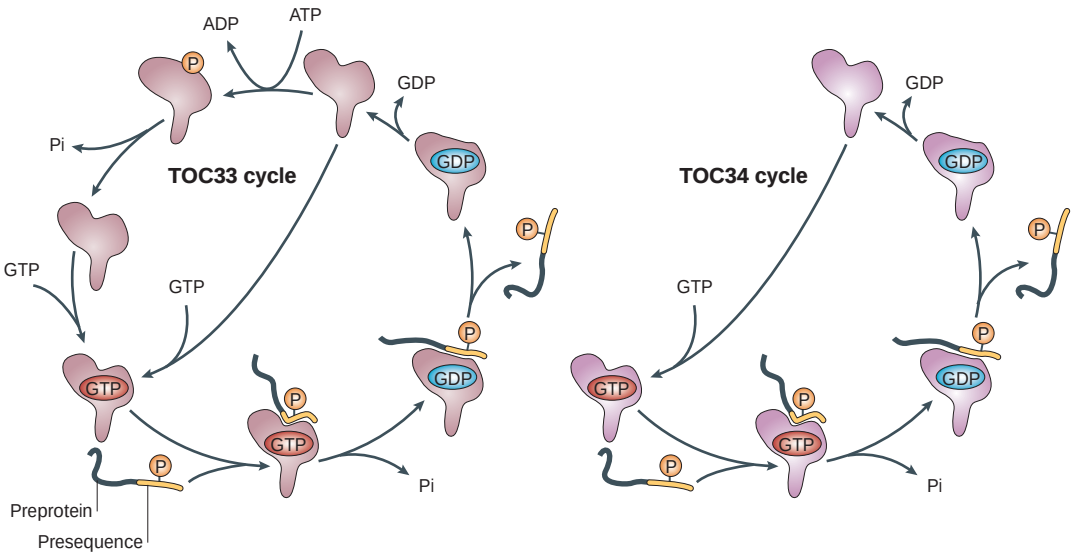


Figure 3 | **Model of the *Arabidopsis thaliana* TOC33 and TOC34 receptor cycles.** The *Arabidopsis thaliana* (*At*)TOC33 (translocon of the outer chloroplast envelope-33) and *At* TOC34 receptors are activated by GTP binding. *At* TOC33/34–GTP binds the phosphorylated preprotein with high affinity. The preprotein activates the endogenous GTPase activity of *At* TOC33/34, which results in the hydrolysis of GTP. *At* TOC33/TOC34–GDP has a lower affinity for the preprotein, which is released to the next translocon subunit. After the release of GDP from *At* TOC33/34, the receptor can be recharged with GTP and enter a new receptor cycle. Alternatively, the *At* TOC33 receptor — which seems to be the functional analogue of the *Pisum sativum* (*Ps*) TOC34 receptor — can be turned off by phosphorylation, which inhibits GTP binding. Dephosphorylation of *At* TOC33 and *Ps* TOC34 is required for re-activation through GTP binding. The reaction cycles for a single *At* TOC 33 and a single *At* TOC34 are shown. Pi, inorganic phosphate. Modified with permission from REF. 21 © (2002) Elsevier.

has a pale-green phenotype and retarded chloroplast development. However, later in development, plants partially recover and are able to grow photoautotrophically on soil. This phenotype is probably due to the presence of a homologue of *At* TOC33 in this organism, that is, *At* TOC34 (REFS 48,50). Expression studies indicate that *At* TOC34 is constitutively expressed at low levels in all organs, whereas *At* TOC33 is upregulated in MERISTEMATIC and rapidly expanding leaf tissue[48,50]. In line with these data is the observation that *At* TOC33 is dispensable for root development[49]. Together, the *in vivo* and *in vitro* results indicate that *At* TOC33 and *At* TOC34 have partially redundant functions. However, both receptors show clear preferences for certain classes of preproteins[47,48,50]. Finally, the different post-translational regulation of *At* TOC33 and *At* TOC34 must be kept in mind. As the *At* TOC33 receptor can be switched off by phosphorylation, the amount of *At* TOC33 protein does not represent the amount of active protein[47].

How do plants respond to the inactivation of genes that encode translocon subunits? Only a limited amount of data are available, which makes it difficult to draw a comprehensive picture. However, the most details are available for TOC33/TOC34 (REF. 51). In *ppi1* knockout plants, the nuclear genes that encode thylakoid-localized photosynthetic proteins tend to be downregulated, whereas genes that encode the proteins involved in carbon metabolism are upregulated. Genes that encode stress proteins — such as HSP70 and the chaperonin-60 system — are also upregulated, which indicates that the chloroplasts experience an overall stress. The transcription of the TOC and TIC subunits is slightly upregulated. Surprisingly, though, the gene that encodes *At* TOC34 is downregulated in *ppi1* plants[51], although artificial overexpression of *At* TOC34 in a *ppi1* background can completely reverse the *ppi1* phenotype[50].

TOC159. TOC159 is a second GTP-binding subunit of the translocon[28,29]. The full-length protein can be divided into three regions: an amino-terminal A-domain, which is rich in acidic amino acids; a central G-domain, which contains the GTP-binding site and is highly similar to the corresponding region in TOC34; and finally the carboxy-terminal membrane or M-domain[52–54]. A small family of TOC159-like proteins is present in *A. thaliana*[53], and it comprises TOC159, TOC132, TOC120 and TOC90. The A-domain becomes progressively smaller as the molecular size of the family members decreases and it is finally absent from *At* TOC90. The G-domain and M-domain are conserved between members of the family[53]. It has therefore been speculated that the A-domain defines certain substrate selectivities, whereas the G- and M-domains represent the more conserved or catalytic properties of this translocon subunit[52,53]. From *in vitro* experiments, we have evidence for two functions of TOC159 (REF. 41). First, it recognizes preproteins in a GTP-dependent manner, which is consistent with its proposed role as a receptor protein. Second, it functions in translocation. The 52-kDa M-domain of

MERISTEMATIC
Actively dividing plant issue.

TOC159 is close to the preprotein[35], which indicates that TOC159 is part of the translocation pore or that it might also contact the preprotein on the inter-envelope-space side of the outer envelope. In addition, TOC159 seems to provide the driving force for membrane translocation[41] (see below). TOC159 is the most prominent phosphoprotein in the outer envelope of *P. sativum* chloroplasts[46], so it might be subject to a similar type of regulation as TOC34.

A soluble form of TOC159 has been detected in the supernatant of mechanically ruptured *P. sativum* leaves[54]. Furthermore, overexpression of a TOC159–GFP (green fluorescent protein) fusion protein in *A. thaliana*, which was under the control of the strong heterologous CaMV 35S-promotor, led to the presence of the fusion protein in the cytosol. TOC159 has therefore been proposed to function as a soluble receptor that shuttles between the cytosol, where it picks up its cargo (preproteins), and the chloroplast surface, where it delivers its cargo to the TOC translocon[54]. Direct experimental support for this idea is lacking. However, the insertion of soluble TOC159 into the outer envelope is facilitated by interaction with TOC34, which could provide a mechanism for TOC-complex assembly and insertion of a receptor–preprotein shuttle[55–57]. Furthermore, Hwang and colleagues made stable complementation lines of the *ppi2* mutant, which is defective in TOC159, using either full-length *At* TOC159 fused to GFP or the M-domain of *At* TOC159 fused to a T7 reporter tag (both were under the control of the CaMV 35S-promoter)[58]. Under the conditions used, they did not observe a soluble form of the *At* TOC159 M-domain — the fusion protein was localized exclusively to the outer envelope. However, the full-length fusion protein had a dual location — it partitioned between the chloroplast surface and the cytosol[58]. As already noted for mitochondria, even mild overexpression can result in protein-import failure and can therefore lead to mislocalization to other cellular compartments[59]. Our own data indicate that the soluble form of TOC159 is associated with chloroplast-specific lipids, which could indicate that TOC159 is present in low-density membrane fragments[97].

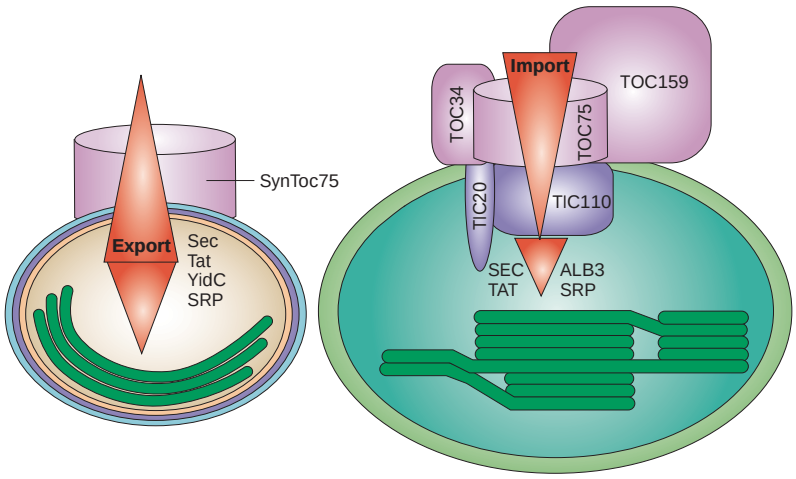
The *ppi2*-mutant phenotype[52] is much more severe than that of *ppi1*, the *At toc33* knockout. The mutant lines have an albino phenotype — leaf plastids do not differentiate into chloroplasts (that is, no thylakoid formation occurs), and the plants are unable to grow on soil and die at the COTYLEDON STAGE[52]. The typical nuclear-encoded gene products that are required for photosynthesis — such as the light-harvesting chlorophyll a/b-binding protein and the small subunit of Rubisco — are dramatically downregulated in *ppi2* plastids[52]. This indicates that other members of the TOC159 family, such as TOC132 or TOC120, cannot fully replace TOC159 and have specialized functions in organelle biogenesis, for example, in the import of non-photosynthetic gene products[52]. The levels of *At* TOC75 and *At* TIC110 in *ppi2* mutants are unaffected, while the transcription of *At toc34* is enhanced[52]. How the regulation of *At* TOC33, which is the predominant isoform in developing chloroplasts, is affected is unknown[52]. Chloroplasts from *ppi2* plants contain very low levels of typically imported photosynthetic proteins — the residual import capacity is insufficient to support proper chloroplast development and results in the death of the seedling[52]. Whether chloroplasts from *ppi2* plants can actually import non-photosynthetic gene-product precursors or contain levels of these polypeptides that are comparable to those in wild-type plants is not known[52].

Complementation of *ppi2* plants with different TOC159 constructs showed that both the G- and M-domain are required to recover a wild-type-like phenotype, whereas the A-domain is dispensable[58]. Plants complemented with the M-domain alone had a greenish phenotype, which indicates that the G-domain is required for full restoration of function. In the absence of the G-domain, plants were yellowish, which again indicates that the M- and A-domains are not sufficient for complementation and that the highly charged A-domain exerts an inhibitory effect[58]. Similar results were obtained by transiently expressing different deletion constructs of *At* TOC159 and simultaneously studying the import of the presequence of the Rubisco small subunit fused to GFP. The deletion constructs containing the G- and M-domain together or the M-domain alone resulted in complete complementation, whereas in the absence of the G-domain — that is, when the A- and M-domains were fused — preprotein import was slow and some preproteins accumulated in the cytoplasm. This again indicated that the A-domain exerts an inhibitory effect on import in this mutant[58]. Together, these data indicate that the M-domain is probably essential, not only for TOC-complex assembly, but also as a functional part of the translocon. The G-domain is required for full complementation, as it constitutes part of the receptor and motor function. The role of the G-domain should become more obvious in kinetic studies, in which it will be possible to compare import rates rather than just a single endpoint after a long incubation time. From *in organello* studies, it is clear that removal of the G-domain still allows residual (by-pass) import under the appropriate conditions (for example, a high ATP concentration and a long incubation time)[60–62]. In these situations, the import yield can be up to 20% of the normal yield.

TOC75. TOC75 is the most abundant outer-envelope protein. Structural analysis of TOC75 predicts that it forms a β-barrel-type channel that, according to computer predictions, is lined by 16 transmembrane β-sheets[63]. Heterologously expressed TOC75 forms a cation-selective, high-conductance ion channel when it is reconstituted into planar lipid bilayers[64]. Electrophysiological measurements indicated that the channel is ~25 Å wide at the entrance, and constricts to ~15–17 Å wide inside the channel. A width of 15 Å could accommodate a polypeptide stretch that still retains some secondary structure[65]. The electrophysiological data also showed that TOC75 has a cytosolic

COTYLEDON STAGE
This stage corresponds to the appearance of the first 'leaves' that emerge during germination of the embryo.

Box 2 | Endosymbiotic origin of chloroplast translocon subunits



Endosymbiosis was accompanied by massive gene transfer from the endosymbiont to the host nucleus. However, before genes could be eliminated from the endosymbiont genome, a system to import the now nuclear-encoded gene products into the new organelle had to be established. Although the endosymbiotic bacterium had several systems to export (or secrete) proteins across the membranes, the organelle now had to import proteins (see figure).

Most striking is the homology of the translocon of the outer-chloroplast-envelope subunit TOC75 to bacterial outer-membrane proteins that are involved in the transport of polypeptides across the outer membrane of Gram-negative bacteria[95,96]. This conserved β-barrel, bacterial-type channel now forms the outer-chloroplast-envelope import channel. The TOC75 homologue in cyanobacteria, SynToc75 seems to be indispensable for growth[67,68]. A β-barrel ion channel has, in most cases, no strong preference for the direction of ion permeation and therefore represents an ideal starting point to build a translocon. Subunits that convey the specificity and directionality of transport are eukaryotic additions, for example, the TOC34 receptor and the TOC159 motor.

But, what formed the translocon of the inner chloroplast envelope (TIC)? There is no detectable homologue for the putative TIC110 channel, and the putative second channel subunit TIC20 shows only a low homology to bacterial proteins. Maybe the early endosymbiont continued to use bacterial export systems in reverse, such as the secretory pathway (SEC), the twin-arginine translocon (TAT) system or the albino3 (ALB3) homologue YIDC[8,10]. Therefore, the TIC translocon — including the adaptation of chaperones in the stroma to provide the driving force for import — could have been an invention of the endosymbiont. Gram-negative bacteria, including cyanobacteria, use the Sec or the Tat system, YidC and an SRP (signal-recognition particle)-dependent pathway to translocate proteins into and across the plasma membrane and the thylakoid membranes. All these systems are still operational in chloroplasts today and are essential for thylakoid biogenesis.

preprotein-binding site, which is able to distinguish between the precursor protein and the mature form[34,64,65]. The binding affinity of TOC75 for preproteins is, however, lower than that of TOC34 or TOC159.

A number of TOC75 isoforms are present in *A. thaliana*, and they are named according to the chromosomal location of the genes[40]. *At* TOC75III is the most abundant outer-envelope protein and is present in the isolated TOC core complex[39]. A viable *At toc75III*-knockout mutant has not been reported so far, which could indicate an essential role for this protein in chloroplast biogenesis. *At* TOC75V is present in the outer envelope with an abundance of less than 10% that of *At* TOC75III (REF. 66). It is not present in the TOC core complex, which could indicate that *At* TOC75V forms a specialized subset of import translocons. *At* TOC75V is homologous to proteins in the outer membrane of Gram-negative bacteria (BOX 2), and it probably represents the most ancestral form of an organelle protein-import channel[66–68]. It is not clear whether *At* TOC75I and *At* TOC75IV are expressed at all in plants, because no expressed-sequence-tag clones have been found so far[40].

TOC64. The role of TOC64 is less well-defined. It has three exposed TETRATRICOPEPTIDE MOTIFS on the cytosolic face of the organelle[38], which are involved in protein–protein interactions. The peroxisomal import receptor PEX5 and the mitochondrial import receptor TOM70 have similar motifs[3,4]. TOM70 functions as a receptor for hydrophobic preproteins (such as carrier proteins that have several internal transport signals), which arrive at the organelle surface as a preprotein–HSP70 complex. The HSP70 chaperone docks onto a specialized tetratricopeptide-repeat domain of the TOM70 import receptor and catalyses the productive transfer of the preprotein to the translocon[69,70]. A similar role could be envisioned for TOC64.

Reconstitution of TOC translocation. In vitro reconstitution experiments represent a useful tool to define more precisely the sequence of events that lead from recognition to translocation, and to characterize single subunits biochemically. The TOC core complex can be isolated from purified envelope membranes as a functionally active unit[33,71]. Analysis of the complex by transmission electron microscopy shows a particle with a diameter of ~130 Å, and a three-dimensional reconstruction map indicates a central finger-like region that separates four curved translocation channels[39] (see the inset in FIG. 2). Reconstitution of this TOC core complex into liposomes showed that it is fully import competent — that is, it could recognize and translocate a preprotein across the liposomal membrane in a GTP-dependent manner[41]. No other nucleoside triphosphate could replace the GTP requirement. Further dissection of this system using single purified TOC subunits showed that TOC34 and TOC159, but not TOC75, can function as GTP-dependent preprotein-binding proteins, that is, receptors. In addition, the reconstitution of TOC75 together with TOC159, but not with TOC34, resulted in preprotein import into a lipid micelle in a GTP-dependent manner[41]. The hydrolysis of GTP by TOC159 is therefore the sole driving force for preprotein translocation *in vitro*. GTP hydrolysis by TOC159 might provoke a conformational change that, in a 'sewing machine'-type mechanism, pushes the preprotein through the TOC75 channel (FIG. 4). TOC75 and TOC159 form the minimal translocon unit *in vitro* that is able to specifically recognize and translocate chloroplast preproteins across a membrane. TOC34 could therefore represent the initial receptor for incoming preproteins. The transfer of the preprotein from TOC34 to TOC159

TETRATRICOPEPTIDE MOTIF
A loosely conserved domain of 30–40 amino acids that is involved in protein–protein interactions.

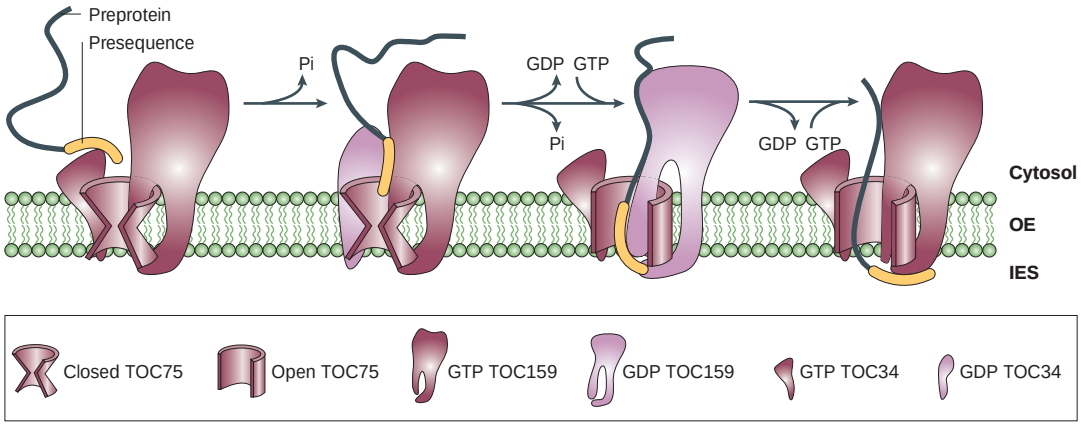


Figure 4 | **Working hypothesis for the action of TOC159.** A preprotein binds to translocon of the outer chloroplast envelope-34 (TOC34)–GTP and, on GTP hydrolysis, this preprotein is transferred to TOC159–GTP. TOC34 then releases GDP and rebinds GTP. In an action that also requires GTP hydrolysis, TOC159 then pushes the preprotein through the TOC75 import channel. A conformational change in TOC159 that is caused by GTP hydrolysis might move the preprotein forward into the channel in a 'sewing machine'-type mechanism. TOC159 then releases GDP and rebinds GTP. Although the TOC core complex is shown in the figure, the minimal complex that is needed for preprotein translocation across a membrane is composed of just TOC75 and TOC159. The gating mechanism of TOC75 is unknown at present. IES, inter-envelope space; OE, outer envelope; Pi, inorganic phosphate.

might be facilitated by the formation of a heterodimer. The recently obtained crystal structure of TOC34 (REF. 72) indicates that it can form homo-oligomers. Due to the extensive sequence similarity between TOC34 and TOC159 (REF. 28), hetero-oligomerization might also be possible[56,57], which would clearly facilitate a smooth transfer of preproteins from one subunit to another. Homotypic interactions between TOC34 and TOC159 occur preferentially when both proteins are in their GDP-bound form[73]. One could speculate that the interaction between TOC34 and TOC159 triggers a GDP-to-GTP exchange in TOC159, which would result in the transfer of the preprotein from TOC34–GDP to TOC159–GTP and subsequently in the dissociation of the TOC34–TOC159 heterodimer (not shown in FIG. 4). The GTP-regulated interaction of translocon subunits is also verified by co-translational protein-translocation systems in bacteria and eukaryotes[74].

The TIC translocon

In most cases, proteins probably translocate through the TOC and TIC complexes simultaneously, although the TIC complex has the ability to retrieve and translocate preproteins that have been released into the inter-envelope space by the TOC translocon[75]. The translocation of preproteins across the inner-envelope membrane requires ATP hydrolysis[76–78], and this ATP is probably needed for the action of molecular chaperones in the stroma, which provide the driving force to complete import into the organelle[79,80]. A membrane potential is not required for any step of the import process[76–78]. Several TIC subunits have been identified, and these are TIC110, TIC62, TIC55, TIC40, TIC22 and TIC20 (FIG. 2).

TIC110, TIC62 and TIC55. TIC110 has one or two hydrophobic transmembrane α-helices in its amino-terminal region[79,81], which are important to target and anchor the protein in the inner-envelope membrane[81]. The topology of the large 80-kDa carboxy-terminal region is not completely resolved (see below). TIC110 is the most abundant and best studied TIC subunit[79–82]. On the inter-envelope-space side, it seems to be in the close vicinity of, or even in contact with, the TOC translocon, because TIC110 often co-fractionates with the TOC complex[32]. On the stromal side of the inner envelope, TIC110 could interact with molecular chaperones such as HSP93 and chaperonin-60 (REFS 79,80). Whether such interactions occur directly or through other TIC subunits remains to be established. Reconstitution experiments using heterologously expressed protein showed that TIC110 can form a cation-selective channel in lipid bilayers[83]. The electrophysiological properties of this channel indicate a pore size of ~15–20 Å, which is similar to the size estimated for TOC75 and is sufficient to allow the passage of a polypeptide chain. Whether further proteins, such as TIC20, participate in channel formation *in vivo* is unknown at present[84].

The exact function of TIC110 is, however, not fully understood at present. Topological studies that assessed the protease accessibility of TIC110 in either intact chloroplasts or isolated inner-envelope-membrane vesicles gave conflicting results. Studies in our laboratory found that TIC110 was accessible to proteases from the inter-envelope space, which is indicative of an exposed domain or multiple transmembrane spans that are connected by protease-accessible loops[81]. Keegstra and colleagues, however, observed that TIC110 was inaccessible to proteases from the inter-envelope space, and therefore concluded that TIC110 exposes a large domain to the chloroplast stroma[85]. Circular dichroism (CD) spectra of heterologously expressed soluble TIC110 (residues 93–966) indicated a largely α-helical conformation[86], whereas CD spectra recorded after the

refolding of a similar TIC110 domain following treatment with 8 M urea indicated a high propensity for β-strands[83]. Clearly more work is needed to settle this important issue.

The TIC complex that was purified by blue-native polyacrylamide gel electrophoresis has an apparent molecular weight of ~250 kDa (REF. 87). Abundant subunits in this complex are TIC110, TIC62 and TIC55. The latter two have the potential to catalyse electron-transfer reactions[87,88]. TIC55 contains a IRON–SULPHUR CENTRE and a mononuclear iron-binding site[87]. TIC62 has a conserved NAD(P)-binding site. The carboxyl terminus of TIC62 is exposed to the stroma and interacts with ferredoxin NAD(P) reductase (FNR; FIG. 2), as deduced from yeast two-hydrid studies and affinity-chromatography experiments[88]. FNR couples photosynthetic electron flux to the reduction of NAD(P)H. NAD(P)H is the primary reductant in CO_2 fixation, nitrogen and sulphur reduction, as well as in fatty-acid and isoprenoid biosynthesis. The interaction between FNR and TIC62 might link the metabolic status of the chloroplast — that is, the NAD(P)H:NAD(P) ratio — to the import capacity of the TIC complex. Support for this idea comes from import experiments that used different ferredoxin isoforms[89]. Ferredoxin I, which is involved in photosynthetic electron flux, is imported both in light and dark conditions. Ferredoxin III, which is more involved in metabolic processes, is mistargeted in the light to the inter-envelope space. Only in the dark is ferredoxin III correctly imported into the stroma[89]. Together, the available data indicate a clear potential for the redox regulation of preprotein import through the TIC complex. It is probable that not all preproteins are sensitive to this control, or that there are distinct TIC subtranslocons that have different regulatory properties.

TIC40. TIC40 is an integral membrane component of the TIC complex and it seems to be tightly associated with TIC110 (REFS 90,91). The carboxy-terminal region is exposed on the stromal site of the inner envelope, and this region seems to have two functional domains. First, it is homologous to HSP70-interacting proteins as well as to HSP70/HSP90-organizing proteins[90] and, second, it has a tetratricopeptide domain for protein–protein interactions[91]. Immunoprecipitation experiments indicate not only a close association with TIC110, but also with stromal HSP93 (REF. 91).

TIC40 is encoded by a single gene in *A. thaliana*[40]. *A. thaliana* plants that contain a T-DNA insertion in the *At tic40* gene have a pale-green phenotype[91]. Their flowering is retarded and their thylakoid grana stacks are not as pronounced as they are in wild-type plants. Chloroplasts isolated from *At tic40*-knockout plants bind preproteins with an efficiency similar to that of wild-type plants; however, translocation into the stroma is reduced by 50% (REF. 91). These results support the proposed role for TIC40 in recruiting molecular chaperones to the TIC translocon, which concentrates these chaperones at, and coordinates chaperone action with, the sites of import[90].

IRON–SULPHUR CENTRE
Iron ions that are complexed by inorganic sulphur and by the amino-acid cysteine function as redox elements in electron-transfer reactions.

TIC22. TIC22 is localized to the inter-envelope space and is only loosely bound to the inner-envelope membrane[84]. It might have a role in the coordination of TOC and TIC functions, or in the guidance of preproteins across the inter-envelope space.

TIC20. TIC20 is an integral protein of the inner-envelope membrane and it has four putative transmembrane α-helices[84]. Due to its similarities to bacterial amino-acid transporters and to the mitochondrial import component TIM17 (REF. 92), it was proposed to participate in the formation of the TIC import channel[84]. However, no *in vitro* data support this idea so far.

The importance of TIC20 for chloroplast biogenesis was studied using an antisense approach[93]. Two genes — *At tic20I* and *At tic20V* — encode the TIC20 isoforms (REF. 40). *A. thaliana* plants that were treated with antisense RNA against *At tic20I* showed reduced levels (between 20–50%) of *At* TIC20I protein compared to wild-type plants. Plants were viable on soil, but they were pale and the chloroplast ultrastructure showed impaired thylakoid formation[93]. Preprotein import into chloroplasts that were isolated from these antisense plants was inhibited by 50%, although preprotein binding to TOC receptors remained normal, which indicates a selective defect in translocation across the inner membrane[93]. The expression of *At tic20IV* and the role of this isoform in antisense-*At-tic20I*-treated plants were not studied, but *At* TIC20IV might help to maintain a viable phenotype in these plants[93]. Although there were changes in the expression and protein levels of *At* TIC20I in plants treated with antisense *At tic20I*, no significant changes in the amount of the other TOC and TIC subunits could be detected[93].

Further insights into the transcriptional control of translocon subunits has come from an independent genetic screen that searched for protein-import mutants. In this screen, a leaf-specific transcription factor was isolated that specifically upregulates *At toc33* and *At toc75III* gene expression[94]. Mutant plants showed only basal transcription rates of the two genes, which were not sufficient to produce enough translocon subunits for normal chloroplast biogenesis. Consequently, these plants had a pale-green phenotype, but could still grow photoautotrophically. Understanding the developmental and organ-specific control of the expression of translocon subunits should, in the future, enable us to predict translocon composition, selectivity and regulation within different plastid types.

Conclusion and perspectives

Protein import into chloroplasts is more complex than was previously anticipated. Regulatory circuits seem to operate at three different levels — in the cytosol and at the TOC and TIC translocons. GTP-dependent regulation at the TOC complex superficially resembles the signal-recognition-particle–Sec61 system. However, the actual import mechanism is a hybrid machine that uses a GTP-dependent motor to move proteins across the outer envelope, and it might be similar to the bacterial

SecA-dependent system, which translocates proteins across the inner bacterial membrane through the SecYEG translocon. The TIC translocon probably uses molecular chaperones as the driving force for translocation and, in this respect, resembles the mitochondrial import system. Although most preproteins seem to use a general import pathway, the unexpected number of genes for isoforms of the TOC and TIC subunits seems to tell a different story. Clearly the protein composition of these translocons in different organs needs to be determined to verify the expression profiles. Biochemical and biophysical methods must be used in reconstituted systems to pinpoint differences in the specificity and regulation of translocon subunits, as well as to understand the cooperation between them. Owing to its inborn complexity, the *in vivo* system is unable to tell us everything. Finally, our efforts to determine the structures of single subunits and of entire complexes should help us to define the mechanistic basis of protein import into chloroplasts.

1. Cavalier-Smith, T. Membrane heredity and early chloroplast evolution. *Trends Plant Sci.* **5**, 174–182 (2000).
2. Rujan, T. *et al*. Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc. Natl Acad. Sci. USA* **99**, 429–441 (2002).
3. Pfanner, N. & Geissler, A. Versatility of the mitochondrial protein import machinery. *Nature Rev. Mol. Cell. Biol.* **2**, 339–349 (2001).
4. Kunau, W. H. Peroxisomes: the extended shuttle to the peroxisome matrix. *Curr. Biol.* **11,** R659–R662 (2001).
5. Neuhaus, J. & Rogers, J. C. Sorting of proteins to vacuoles in plant cells. *Plant Mol. Biol.* **38**, 127–144 (1998).
6. Leister, D. Chloroplast research in the genomic age. *Trends Genet.* **19**, 46–47 (2003).
7. Race, H. L., Herrmann, R. G. & Martin, W. Why have organelles retained genomes? *Trends Genet.* **15**, 364–370 (1999).
8. Mori, H. & Cline, K. Post-translational protein translocation into thylakoids by the Sec and ΔpH-dependent pathways. *Biochim. Biophys. Acta* **1541**, 80–90 (2001).
9. Vothknecht, U. & Westhoff, P. Biogenesis and origin of thylakoid membranes. *Biochim. Biophys. Acta* **1541**, 91–101 (2001).
10. Eichacker, L. A. & Henry, R. Function of a chloroplast SRP in thylakoid protein export. *Biochim. Biophys. Acta* **1541**, 120–134 (2001).
11. Van Dooren, D. D., Schwartzbach, S. D., Osafine, T. & McFadden, G. I. Translocation of proteins across the multiple membranes of complex plastids. *Biochim. Biophys. Acta* **1541**, 34–53 (2001).
12. Svesnikova, N., Soll, J. & Schleiff, E. Toc34 is a preprotein receptor regulated by GTP and phosphorylation. *Proc. Natl Acad. Sci. USA* **97**, 4973–4978 (2000).
13. Young, M. E., Keegstra, K. & Froehlich, J. E. GTP promotes the formation of early-import intermediates but is not required during the translocation step of protein import into chloroplasts. *Plant Physiol.* **121**, 237–243 (1999).
14. Jackson-Constan, D., Akita, M. & Keegstra, K. Molecular chaperones involved in chloroplast protein import. *Biochim. Biophys. Acta* **1541**, 102–113 (2001).
15. Attardi, G. & Schatz, G. A. Biogenesis of mitochondria. *Annu. Rev. Cell Biol.* **4**, 289–333 (1988).
16. Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. & Schekmann, R. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature* **332**, 800–805 (1988).
17. Zimmermann, R., Sagstetter, M., Lewis, M. J. & Pelham, H. R. B. Seventy-kDa heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes. *EMBO J.* **7**, 2875–2880 (1988).
18. Waegemann, K., Paulsen, H. & Soll, J. Translocation of proteins into isolated chloroplasts requires cytosolic factors to obtain import competence. *FEBS Lett*. **261**, 89–92 (1990).
19. Keegstra, K. & Froehlich, J. E. Protein import into chloroplasts. *Curr. Opin. Plant Biol.* **2**, 471–476 (1999).
20. Bauer, J., Hiltbrunner, A. & Kessler, F. Molecular biology of chloroplast biogenesis: gene expression, protein import and intraorganellar sorting. *Cell Mol. Life Sci.* **58**, 420–433 (2001).
21. Soll, J. Protein import into chloroplasts. *Curr. Opin. Plant Biol.* **5**, 529–535 (2002).
22. Chen, X. & Schnell, D. J. Protein import into chloroplasts. *Trends Cell Biol.* **9**, 222–227 (1999).
23. Bruce, B. D. The paradox of plastid transit peptides: conservation of function despite divergence in primary structure. *Biochim. Biophys. Acta* **1541**, 2–21 (2001).
24. Miras, S. *et al*. Non-canonical transit peptide for import into the chloroplast. *J. Biol. Chem.* **277**, 47770–47778 (2002).
An example of inner-chloroplast-envelope proteins that lack a cleavable transit peptide and are still targeted to chloroplasts.
25. Emanuelsson, O. & von Heijne, G. Prediction of organellar targeting signals. *Biochim. Biophys. Acta* **1541**, 114–119 (2001).
26. Waegemann, K. & Soll, J. Phosphorylation of the transit sequence of chloroplast precursor proteins. *J. Biol. Chem.* **271**, 6545–6554 (1996).
Shows that different chloroplast preproteins contain a common phosphorylation site for a cytosolic protein kinase.
27. May, T. & Soll, J. 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. *Plant Cell* **12**, 53–63 (2000).
28. Kessler, F., Blobel, G., Patel, H. A. & Schnell, D. J. Identification of two GTP-binding proteins in the chloroplast protein import machinery. *Science* **266**, 1035–1039 (1994).
Contains the initial description of TOC34 and TOC159.
29. Hirsch, S., Muckel, E., Heemeyer, F., von Heijine, G. & Soll, J. A receptor component of the chloroplast protein translocation machinery. *Science* **266**, 1989–1992 (1994).
30. Seedorf, M., Waegemann, K. & Soll, J. A constituent of the chloroplast import complex represents a new type of GTP-binding protein. *Plant J.* **7**, 401–411 (1995).
31. Perry, S. E. & Keegstra, K. Envelope membrane proteins that interact with chloroplastic precursor proteins. *Plant Cell* **6**, 93–105 (1994).
Used a chemical-crosslinking approach to identify chloroplast translocon subunits.
32. Schnell, D. J., Kessler, F. & Blobel, G. Isolation of components of the chloroplast protein import machinery. *Science* **266**, 1007–1012 (1994).
33. Waegemann, K. & Soll, J. Characterisation of the protein import apparatus in isolated outer envelopes of chloroplast. *Plant J.* **1**, 149–159 (1991).
Established that isolated outer-envelope membranes are a bona fide system to study the early steps in protein translocation.
34. Ma, Y., Kouranov, A., LaSala, S. & Schnell, D. J. Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope. *J. Cell. Biol.* **134**, 315–327 (1996).
35. Kouranov, A. & Schnell, D. Analysis of the interaction of preproteins with the import machinery over the course of protein import into chloroplasts. *J. Cell Biol.* **139**, 1677–1685 (1997).
36. Lübeck, J. & Soll, J. Nucleoside diphosphate kinase from pea chloroplasts: purification, cDNA cloning and import into chloroplasts. *Planta* **196**, 668–673 (1995).
37. Tranel, P. J., Froehlich, J., Goyal, A. & Keegstra, K. A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. *EMBO J.* **14**, 2436–2446 (1995).
38. Sohrt, K. & Soll, J. Toc64, a new component of the protein translocon of chloroplasts. *J. Cell Biol.* **148**, 1213–1221 (2000).
39. Schleiff, E., Soll, J., Küchler, M., Kühlbrandt, W. & Harrer, R. Characterisation of the translocon of the outer envelope of chloroplasts. *J. Cell. Biol.* **160**, 541–551 (2003).
40. Jackson-Constan, D. & Keegstra, K. *Arabidopsis* genes encoding components of the chloroplastic protein import apparatus. *Plant Physiol.* **125**, 1567–1576 (2001).
41. Schleiff, E., Jelic, M. & Soll, J. A GTP-driven motor moves proteins across the outer envelope of chloroplasts. *Proc. Natl Acad. Sci. USA* **100**, 4604–4609 (2003).
Reports the reconstitution of protein translocation in a liposome system using single purified TOC components.
42. Schleiff, E. *et al*. Structural and GTP/GDP requirements for transit peptide recognition by the cytosolic domain of the chloroplast outer envelope receptor, Toc34. *Biochemistry* **41**, 1934–1946 (2002).
43. Jelic, M. *et al*. The chloroplast import receptor Toc34 functions as preprotein-regulated GTPase. *Biol. Chem*. **383**, 1875–1883 (2002).
Showed that endogenous TOC34 GTPase activity is stimulated specifically by preproteins.
44. Scheffzek, K., Ahmadian, M. R., & Wittinghofer, A. GTPase-activating proteins: helping hands to complement an active site. *Trends Biochem. Sci.* **23**, 257–262 (1998).
45. Cherfils, J. & Chardin, P. GEFs: structural basis for their activation of small GTP-binding proteins. *Trends Biochem. Sci.* **24**, 306–311 (1999).
46. Fulgosi, H. & Soll, J. The chloroplast protein import receptors Toc34 and Toc159 are phosphorylated by distinct protein kinases. *J. Biol. Chem.* **277**, 8934–8940 (2002).
47. Jelic, M., Schleiff, E. & Soll, J. Two Toc34 homologues with different properties. *Biochemistry* **42**, 5906–5916 (2003).
Showed that *At* TOC33 and *At* TOC34 are differently regulated and that they have different affinities for certain preproteins.
48. Gutensohn, M., Schulz, B., Nicolay, P. & Flügge, U. Functional analysis of the two *Arabidopsis* homologues of Toc34, a component of the chloroplast protein import apparatus. *Plant J.* **23**, 771–783 (2000).
Used an antisense approach to investigate the roles of *At* TOC33 and *At* TOC34 in plastid biogenesis and import.
49. Yu, T.-S. & Li, H. Chloroplast protein translocon components atToc159 and atToc33 are not essential for chloroplast biogenesis in guard cells and root cells. *Plant Physiol*. **127**, 90–96 (2001).
50. Jarvis. P. *et al*. An *Arabidopsis* mutant defective in the plastid general protein import apparatus. *Science* **282**, 100–103 (1998).
Used T-DNA insertion lines were to elucidate the phenotype of an *At toc33* knockout.
51. Kubis, S. *et al*. The *Arabidopsis ppi1* mutant is specifically defective in the expression, chloroplast import and accumulation of photosynthetic proteins. *Plant Cell* **15**, 1859–1871 (2003).
52. Bauer, J. *et al*. The major protein import receptor of plastids is essential for chloroplast biogenesis. *Nature* **403**, 203–207 (2000).
Describes an *At toc159* T-DNA knockout line, including the resulting phenotype and ultrastructure changes.
53. Hiltbrunner, A., Bauer, J., Alvarez-Huerta, M. & Kessler, F. Protein translocon at the *Arabidopsis* outer chloroplast membrane. *Biochem. Cell Biol.* **79**, 629–635 (2001).
54. Hiltbrunner, A. *et al*. Targeting of an abundant cytosolic form of the protein import receptor at Toc159 to the outer chloroplast membrane. *J. Cell Biol.* **154**, 309–316 (2001).
Proposes that TOC159 functions as a receptor that shuttles between the cytosol and the chloroplast surface.
55. Bauer, J. *et al*. Essential role of the G-domain in targeting of the protein import receptor atToc159 to the chloroplast outer membrane. *J. Cell Biol.* **159**, 845–854 (2002).
56. Weibel, P., Hiltbrunner, A., Brand, L. & Kessler, F. Dimerization of Toc-GTPases at the chloroplast protein import machinery. *J. Biol. Chem.* **278**, 37321–37329 (2003).
57. Wallas, T., Smith, M., Sanchez-Nieto, S. & Schnell, D. The roles of Toc34 and Toc75 in targeting the Toc159 preprotein receptor to chloroplasts. *J. Biol. Chem.* **278**, 44289–44297 (2003).
58. Lee, K. H., Kim, S. J., Lee, X. J. & Hwang, I. The M domain of atToc159 plays an essential role in the import of proteins into chloroplasts and chloroplast biogenesis. *J. Biol. Chem.* **278**, 36794–36805 (2003).

59. Sickmann, A. *et al*. The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc. Natl Acad. Sci. USA* **100**, 13207–13212 (2003).
60. Chen, K., Chen, X. & Schnell, D. Initial binding of preproteins involving the Toc159 receptor can be bypassed during protein import into chloroplasts. *Plant Physiol.* **122**, 813–822 (2000).
61. Friedman, A. & Keegstra, K. Chloroplast protein import — quantitative analysis of precursor binding. *Plant Physiol.* **89**, 993–999 (1989).
62. Cline, K., Werner-Washburne, M., Lubben, T. & Keegstra, K. Precursors to two nuclear-encoded chloroplast proteins bind to the outer envelope membrane before being imported into chloroplasts. *J. Biol. Chem.* **260**, 3691–3696 (1985).
63. Svesnikova, N., Grimm, R., Soll, J. & Schleiff, E. Topology studies of the chloroplast protein import channel Toc75. *Biol. Chem.* **381**, 687–693 (2000).
64. Hinnah, S., Hill, K., Wagner, R., Schlicher, T. & Soll, J. Reconstitution of a chloroplast protein import channel. *EMBO J.* **16**, 7351–7360 (1997).
65. Hinnah, S. C., Wagner, R., Sveshnikova, N., Harrer, R. & Soll, J. The chloroplast protein import channel Toc75: pore properties and interaction with transit peptides. *Biophys. J.* **83**, 899–911 (2002).
Describes the electrophysical characterization of the protein-import channel TOC75.
66. Eckart, K., Eichacker, L., Sohrt, K., Schleiff, E., Heins, L. & Soll, J. A Toc75-like protein import channel is abundant in chloroplasts. *EMBO rep.* **3**, 557–562 (2002).
67. Bölter, B., Soll, J., Schulz, A., Hinnah, S. & Wagner, R. Origin of a chloroplast protein importer. *Proc. Natl Acad. Sci USA* **95**, 15831–15836 (1998).
68. Reumann, S., Davila-Aponte, J. & Keegstra, K. The evolutionary origin of the protein-translocating channel of chloroplastic envelope membranes: identification of a cyanobacterial homologue. *Proc. Natl Acad. Sci. USA* **96**, 784–789 (1999).
69. Young, J. C., Hoogenraad, N. J. & Hartl, F. U. Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. *Cell* **112**, 41–50 (2003).
Characterized the role of the TOM70–HSP70 interaction, which occurs through tetratricopeptide-repeat domains of TOM70, in mitochondrial import.
70. Wiedemann, N., Pfanner, N. & Ryan, M. T. The three modules of ADP/ATP carrier cooperate in receptor recruitment and translocation into mitochondria. *EMBO J.* **20**, 951–960 (2001).
71. Soll, J. & Waegemann, K. A functionally active protein import complex from chloroplasts. *Plant J.* **2**, 253–256 (1992).
72. Sun J.-Y. *et al*. Crystal structure of pea Toc34, a novel GTPase of the chloroplast protein translocon. *Nature Struct. Biol.* **9**, 95–100 (2002).
The first crystal structure of a chloroplast import machinery subunit, TOC34.
73. Smith, M., Hiltbrunner, A., Kessler, F. & Schnell, D. The targeting of the atToc159 preprotein receptor to the chloroplast outer membrane is mediated by its GTPase domain and is regulated by GTP. *J. Cell Biol.* **159**, 833–843 (2002).
74. Driessen, A., Manting, E. & van der Does, C. The structural basis of protein targeting and translocation in bacteria. *Nature Struct. Biol.* **8**, 492–498 (2001).
75. Scott, S. V. & Theg, S. M. A new chloroplast protein import intermediate reveals distinct translocation machineries in the two envelope membranes: energetics and mechanistic implications. *J. Cell Biol.* **132**, 63–75 (1996).
76. Schindler, C., Hracky, R. & Soll, J. Protein transport in chloroplasts: ATP is prerequisite. *Z. Naturforsch.* **42c**, 103–108 (1987).
77. Flügge, U. I. & Hinz, G. Energy dependence of protein translocation into chloroplasts. *Eur. J. Biochem.* **160**, 563–567 (1986).
The initial report that established that ATP hydrolysis is required for preprotein import.
78. Pain, D. & Blobel, G. Protein import into chloroplasts requires a chloroplast ATPase. *Proc. Natl Acad. Sci. USA* **84**, 3288–3292 (1987).
79. Kessler, F. & Blobel, G. Interaction of the protein import and folding machineries of the chloroplast. *Proc. Natl Acad. Sci. USA* **93**, 7684–7689 (1996).
80. Nielsen, E., Akita, M., Davila-Aponte, J. & Keegstra, K. Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. *EMBO J.* **16**, 935–946 (1997).
81. Lübeck, J., Soll, J., Akita, M., Nielsen, E. & Keegstra, K. Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. *EMBO J.* **15**, 4230–4238 (1996).
82. Akita, M., Nielsen, E. & Keegstra, K. Identification of protein transport complexes in the chloroplastic envelope membranes via chemical cross-linking. *J. Cell. Biol.* **136**, 9983–9994 (1997).
83. Heins, L. *et al*. The preprotein conducting channel at the inner envelope membrane of plastids. *EMBO J.* **21**, 2616–2625 (2002).
84. Kouranov, A., Chen, X., Fuks, B. & Schnell, D. J. Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane. *J. Chem. Biol.* **143**, 991–1002 (1998).
85. Jackson, D., Froehlich, J. & Keegstra, K. The hydrophilic domain of Tic110, an inner envelope membrane component of the chloroplastic protein translocation apparatus, faces the stromal compartment. *J. Biol. Chem.* **273**, 16583–16588 (1998).
86. Inaba, T., Li, M., Avarez-Huerta, M., Kessler, F. & Schnell, D. atTic110 functions as a scaffold for coordinating the stromal events of protein import into chloroplasts. *J. Biol. Chem.* **278**, 38617–38627 (2003).
87. Caliebe, A., Grimm, R., Kaiser, G., Lübeck, J., Soll, J. & Heins, L. The chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding protein. *EMBO J.* **16**, 7342–7350 (1997).
Blue-native polyacrylamide gel electrophoresis was used to analyse the composition of the TIC complex.
88. Küchler, M., Decker, S., Soll, J. & Heins, L. Protein import into chloroplasts involves redox-regulated proteins. *EMBO J.* **21**, 6136–6145 (2002).
89. Hirohashi, T., Hase, T. & Nakai, M. Maize non-photosynthetic ferredoxin precursor is missorted to the intermembrane space of chloroplasts in the presence of light. *Plant Physiol.* **125**, 2154–2163 (2001).
90. Stahl, T., Glockmann, C., Soll, J. & Heins, L. Tic40, a new 'old' subunit of the chloroplast protein import translocon. *J. Biol. Chem.* **274**, 37467–37472 (1999).
91. Chou, M.-L. *et al*. Tic40, a membrane-anchored co-chaperone homolog in the chloroplast protein translocon. *EMBO J.* **22**, 2970–2980 (2003).
92. Rassow, J., Dekker, P., van Wilpe, S., Meijer M. & Soll, J. The preprotein translocase of the mitochondrial inner membrane: function and evolution. *J. Mol. Biol.* **286**, 105–120 (1999).
93. Chen, X., Smith, M. D., Fitzpatrick, L. & Schnell, D. J. *In vivo* analysis of the role of atTic20 in protein import into chloroplasts. *Plant Cell* **14**, 641–654 (2002).
94. Sun, C.-W., Chen, L.-J., Lin, L.-C. & Li, H. Leaf-specific upregulation of chloroplast translocon genes by a CCT motif-containing protein, CIA2. *Plant Cell* **13**, 2053–2061 (2001).
Describes the design of a new screen that enabled the authors to select new types of import mutant.
95. Heins, L. & Soll, J. Chloroplast biogenesis: mixing the prokaryotic and the eukaryotic? *Curr. Biol.* **8**, 215–217 (1998).
96. Reumann, S. & Keegstra, K. The endosymbiotic origin of the protein import machinery of chloroplastic envelope membranes. *Trends Plant Sci.* **4**, 302–307 (1999).
Contains a compilation of the genes and gene families that encode the subunits of the TOC and TIC translocons.
97. Becker, T., Jelic, M., Vojta, A., Radunz, A., Soll, J. & Schleiff, E. Preprotein recognition by the Toc complex. *EMBO J.* **23**, 520–530 (2004).

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Competing interests statement

The authors declare that they have no competing financial interests.

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