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# Revaluating the evolution of the Toc and Tic protein translocons

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**The origin of the plastid from a cyanobacterial endosymbiont necessitated the establishment of specialized molecular machines (translocons) to facilitate the import of nuclear-encoded proteins into the organelle. To improve our understanding of the evolution of the translocons at the outer and inner envelope membrane of chloroplasts (Toc and Tic, respectively), we critically reassess the prevalent notion that their subunits have a function exclusive to protein import. We propose that many translocon components are multifunctional, conserving ancestral pre-endosymbiotic properties that predate their recruitment into the primitive translocon (putatively composed of subunits Toc34, Toc75 and Tic110 and associated chaperones). Multifunctionality seems to be a hallmark of the Tic complex, in which protein import is integrated with a broad array of plastid processes.**

## The establishment of plastids required a primitive protein translocon

The plastid originated from a cyanobacterial endosymbiont that was harnessed *circa* 1.5 billion years ago by a heterotrophic, unicellular protist. This event occurred in the putative common ancestor of photosynthetic eukaryotes that comprise the 'supergroup' Plantae (glaucophytes, rhodophytes and chlorophytes [including land plants]) (Figure 1a) [1]. Organelle genesis relied on the 'host' cell gaining increasing control over the cellular functions of the captured prokaryote. This was driven in large part by the massive relocation of endosymbiont genes to the nucleus [1,2]. Progressive genetic and biochemical integration of the endosymbiont into the Plantae ancestor undoubtedly required a new system for protein trafficking that allowed the regulated import of nuclear-encoded proteins into the organelle [1,2]. The evolution of the Toc and Tic protein translocons (translocons at the outer and inner envelope membrane of chloroplasts, respectively) in the primitive organelle represents a remarkable solution to this fundamental problem, allowing modern-day plastids to routinely import >2000 nuclear-encoded, transit peptide (TP)-containing proteins to express their function inside the organelle [3].

The Toc and Tic translocons in plastids of higher plants are protein complexes comprising multiple specialized subunits that function as pre-protein receptors, protein-conducting channels, co-chaperones and even regulatory subunits (Figure 1b, Box 1 and Box 2). Such a level of complexity raises the question of how these marvels of engineering evolved: what was the nature of the minimal functional set of subunits that might have comprised the primitive plastid translocon in the Plantae ancestor? To address this issue, we critically reassess two key prevailing ideas about plastid translocon evolution: (i) Toc and Tic subunits have specialized functions that are exclusive to plastid protein import; and (ii) subunits of the translocon that have a pre-endosymbiotic (i.e. cyanobacterial) origin (Figure 1b) lost their ancestral functions when they were co-opted for plastid protein translocation [4,5]. We evalu-

### Glossary

**Blue native PAGE**: this form of polyacrylamide gel electrophoresis does not utilize SDS and therefore permits the proteins to migrate in the gel in their native state, keeping the original protein complexes intact. Protein complexes are visualized using the blue dye Coomassie.
**Chaperone**: a protein that regulates the folding or unfolding state of a client protein or mediates the assembly or disassembly of multisubunit complexes. Chaperones also provide a driving force for protein translocation across the envelope membranes of chloroplasts. Chaperones are assisted by auxiliary factors called co-chaperones.
**Co-immunoprecipitation**: an antibody raised against a given Toc or Tic subunit is used to directly precipitate its target (by immunoaffinity purification). Any other Toc or Tic subunit that interacts with the primary target will also be indirectly co-precipitated.
**Immunoaffinity purification of an epitope-tagged protein**: a recombinant Toc and Tic protein can be fused with a specific tag that serves as a target (epitope) for antibody recognition. Using chromatography, protein extracts from chloroplast envelope membranes can be passed through a column containing the antibody coupled to a solid support. The antibody will selectively bind (by immunoaffinity) to its epitope and retain the epitope-fused protein and any of its interactors in the column. These can be eluted from the column, maintaining the integrity of the protein complex.
**Pre-protein**: an immature protein containing a TP.
**Protein complex**: two or more polypeptides that physically interact, forming a functional unit.
**Reversible crosslinking**: radiolabelled pre-proteins can be arrested in intermediate states of translocation in close contact with the Toc or Tic machineries (Box 3). By application of a chemical agent, covalent bonds are formed between the radiolabelled pre-proteins and adjacent translocon subunits. The entire 'crosslinked' complex is then purified and the covalent bonds cleaved by another chemical treatment. This releases the individual crosslinking components, which can be separated by SDS-PAGE.
**SDS-PAGE**: sodium dodecyl sulfate polyacrylamide gel electrophoresis is a method for the separation of proteins by electrophoresis using a polyacrylamide gel. The addition of SDS causes the disassociation of protein complexes, allowing each individual protein to migrate separately in the gel according to its molecular size.
**T-DNA**: transferred-DNA is a segment of the tumor-inducing plasmid of *Agrobacterium tumefaciens*. Upon infection of a plant such as *Arabidopsis thaliana* with *Agrobacterium*, T-DNA can be relocated into the nuclear genome of the plant. Insertion of T-DNA in a plant gene causes its disruption and loss of its function (i.e., mutagenesis).
**Transit peptide (TP)**: N-terminal extensions in nuclear-encoded proteins that serve as a signal for plastid targeting and translocation through the Toc and Tic complexes. TPs are removed by a stromal processing peptidase (SPP) in the plastid interior.

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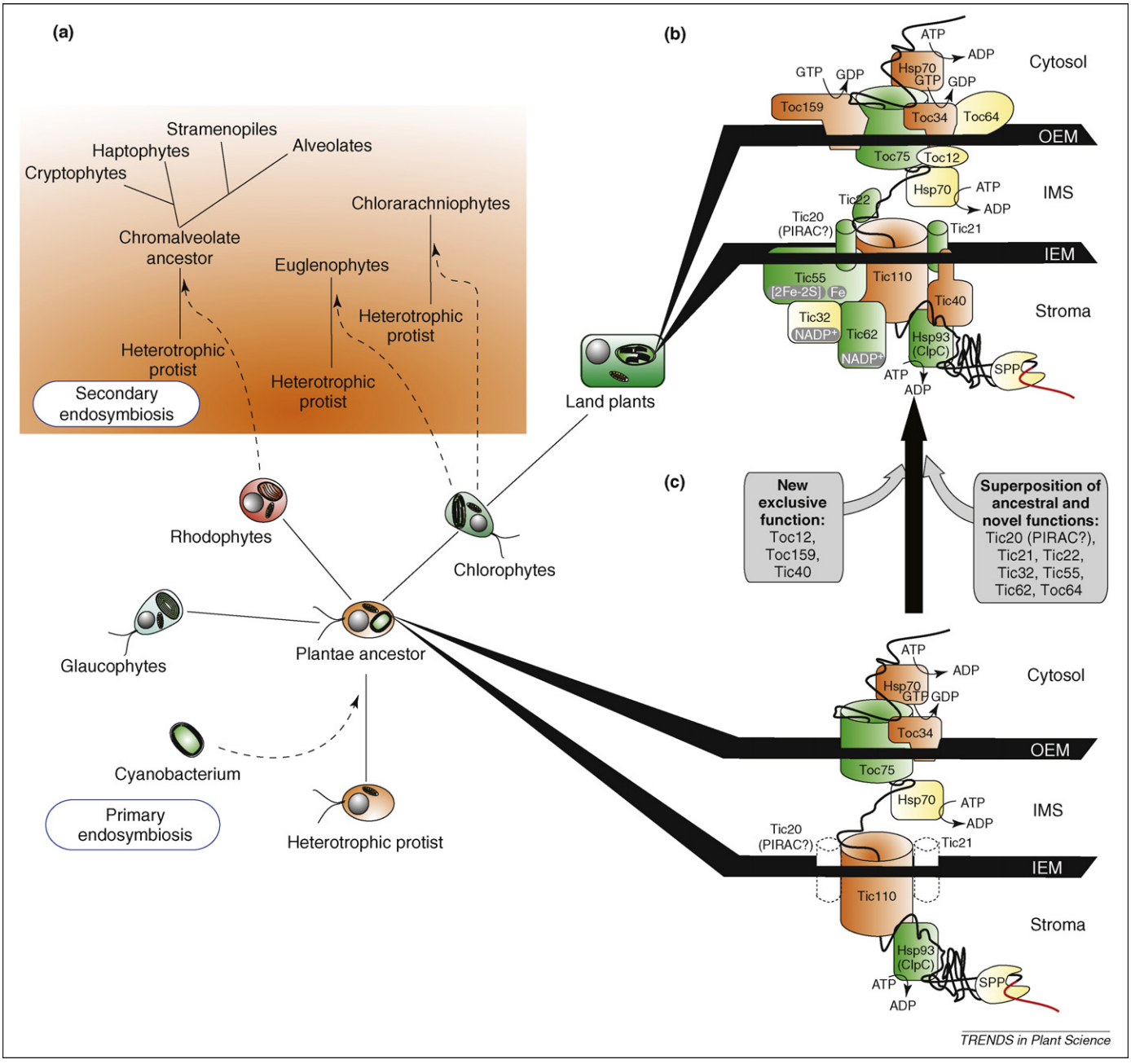


**Figure 1**. The structure and phylogenetic affiliation of the Toc and Tic subunits in plastids of Plantae reflects their origin through primary endosymbiosis. (a) A cyanobacterium was captured by a mitochondriate eukaryote (i.e. a heterotrophic protist) and transformed into the plastid. This primary endosymbiotic event resulted in the ancestor of the algal lineages Chlorophyta, Rhodophyta and Glaucophyta. Land plants are derived from chlorophyte algae. Secondary endosymbiotic events (brown background) resulted in the chromalveolate, euglenophyte and chlorarachniophyte algae when a heterotrophic protist engulfed an existing alga with a 'primary' plastid. Broken lines represent the emergence through primary or secondary endosymbiosis of the eukaryotic plastid. (b) The Toc and Tic translocons of higher plants in the process of importing a pre-protein from the cytosol are shown from the perspective of the chloroplast envelope membranes. The unfolded cytosolic proteins containing a TP (red N-terminal segment) are initially recognized by the receptors Toc34 and Toc159 and then imported across the outer and inner envelope membranes (OEM and IEM, respectively) of plastids through the pore components Toc75 and Tic110. The translocation process is energy dependent (ATP hydrolysis) and is driven by heat shock-type molecular chaperones (Hsp70 and Hsp93) acting at the cytosolic intermembrane space (IMS) and stromal sides of the organelle. A stromal processing peptidase (SPP) proteolytically removes the TP from post-translocated polypeptides. Several subunits are functionally engaged in the Toc and Tic translocons (Box 1, Box 2). The subunits of the translocon shown in green are of cyanobacterial (endosymbiont) origin, those in red are of eukaryotic (host) provenance and the yellow subunits are of unknown phylogenetic affiliation [4,5]. (c) The proposed primitive state of the translocon in the Plantae ancestor was simple and composed of the Toc34, Toc75 and Tic110 subunits and the Hsp70 and Hsp93 chaperones. The hypothetical inclusion (indicated by broken lines) of the Tic20 and Tic21 subunits in the primitive translocon is still uncertain and depends on direct experimental proof of their participation as channel components of the Tic translocon. The black arrow indicates the evolution from the primitive translocon to the modern Toc and Tic complexes found in plant chloroplasts. This transition involved the addition of subunits to fulfil an exclusive new function in protein import (Toc12, Toc159 and Tic40) or subunits that were functionally recruited to the translocon but maintained ancestral properties (listed on the right). We tentatively classify Toc64 in the latter group because it shows sequence conservation with plant amidases (50% identity and 67% similarity over the full sequence of functional amidase 1 (AMI1) in *Arabidopsis*, data not shown), suggesting a putative functional correlation. We raise here the question of whether the protein import-related anion channel (PIRAC) corresponds to Tic20 (Box 1 and main text).

**Box 1. The proposed functions of the Toc and Tic subunits**

**Toc34 and Toc159**
These subunits are GTPases that serve as receptors for TP-containing proteins incoming from the cytosol. Toc34 and Toc159 might act cooperatively, forming a GTPase gate for Toc75. This presumably operates when binding of the pre-protein causes GTP hydrolysis and sequential dimerization of Toc34–Toc159, followed by release of the pre-protein into the Toc75 channel. It is suggested that Toc159 might act as a motor, pushing import substrates through the Toc75 channel [54].

**Toc75**
The protein-conducting channel of the Toc translocon, which can be reconstituted in planar lipid bilayers as a cation selective and high-conductance pore with an internal width of ~15–17 Å and an affinity for pre-proteins [41,42].

**Toc12**
An IMS-oriented subunit that is proposed to act as a co-chaperone for recruiting and activating IMS Hsp70, which is a putative driving motor for protein translocation across the OEM [46].

**Toc64**
A proposed receptor that sequentially interacts with Hsp90-bound import substrates incoming from the cytosol and delivers the pre-proteins to Toc34 [55]. Its actual function in protein translocation is disputed [47,48].

**Tic22**
A small IMS protein that peripherally associates with the Tic complex and interacts with pre-proteins in transit. Tic22 has been proposed to serve as an adaptor that facilitates pre-proteins *en route* from the Toc translocon towards the Tic complex [23].

**Tic110**
The proposed protein-conducting channel of the Tic complex [31] and docking module for stromal components involved in late stages of protein import [32,33,38].

**Tic20**
An integral IEM protein that physically associates with Tic110. This subunit has been traditionally proposed to be a constituent of the IEM protein-conducting channel [23,24].

**Tic21**
This integral protein of the IEM has recently been identified as a *bona fide* Tic component. It is speculated that Tic21 might serve as a protein-conducting channel [21].

**Tic40**
A co-chaperone that, when bound to Tic110, recruits and stimulates the ATP hydrolytic activity of ClpC, which is the translocation motor for protein import across the Tic channel [34,36,37].

**Tic55, Tic62 and Tic32**
Putative regulatory subunits (Box 2).

**PIRAC**
A protein import-related anion channel (PIRAC) that has been consistently associated to the Tic translocon in electrophysiological measurements taken from isolated chloroplasts [28,29]. This component is not considered to be a constituent of the protein import machinery and its molecular nature is still unknown. In this article, we treat PIRAC as a hypothetical subunit of the Tic complex.

ate recent data that bring into question these two views and indicate that many Toc and Tic components possess a functional duality that might reflect conservation of ancestral pre-endosymbiotic properties. This new perspective provides an opportunity to reassess the plastid translocon

**Box 2. Ti55, Tic62 and Tic32 are putative regulatory subunits**

Tic55, Tic62 and Tic32 are localized at the stromal side of the IEM of chloroplasts (Figure I). These subunits are thought to act as regulatory factors that, based on their cofactors, act as biosensors, collect information regarding variations in the redox status of the plastid and accordingly modulate protein import rates through the Tic translocon [6,16–20]. In line with this idea, Tic55 is predicted to use a Rieske-type two-iron-two-sulfur cluster – [2Fe-2S] – and mononuclear iron as putative redox biosensors [6], whereas both Tic62 and Tic32 bind an NADPH cofactor [16,18]. In addition, the C-terminal region of Tic62 (which exists only in vascular plants [14,17]) specifically interacts with ferrodoxin-NAD(P)$^+$ reductase, a key redox component in plastids [16,20]. The function of *P. sativum* Tic55 and a putative role of its cofactors [2Fe-2S] and mononuclear iron were tested in an *in vitro* protein-import experiment (Box 3). This was done by application of diethylpyrocarbonate (DEPC), a chemical inhibitory agent that modifies histidine residues, such as those serving as ligands for Tic55 redox cofactors. Addition of DEPC caused an observable defect in the import of the precursor of the ribulose bisphosphate carboxylase small subunit at the level of the Tic complex [6]. Although this result was interpreted as circumstantial evidence for a function of Tic55 in protein import, the mechanism by which Tic55 modulates the protein-import activity according to plastid redox signals has not yet been experimentally tested. By contrast, the regulatory mode of action of Tic62 and Tic32 is better understood. Under oxidizing conditions in the chloroplast (i.e. a high [NADP$^+$]/[NADPH] ratio), Tic62 and Tic32 increase their association with Tic110 and the IEM fraction of chloroplasts (Figure I). By contrast, under reducing conditions (i.e. a low [NADP$^+$]/[NADPH] ratio), both dissociate from Tic110, becoming soluble in the stroma. The dynamic partitioning of Tic62 and Tic32 pools between Tic110-dissociated or Tic110-associated states might relay information regarding the redox status of chloroplasts and modulate protein-import activity [19,20]. In addition to redox regulation, calcium signalling has also been demonstrated to influence protein import rates in chloroplasts [19]. Tic32 interacts with calmodulin, which is the major calcium sensor in eukaryotic cells. It has been proposed that calmodulin, by sensing high concentrations of calcium in chloroplast, interacts with Tic32, leading to the release of NADPH from the protein. This would increase the pool of NADPH-lacking Tic32, which would shift from its soluble form to association with Tic110, causing the postulated regulation of the protein-import rates in response to calcium [19].

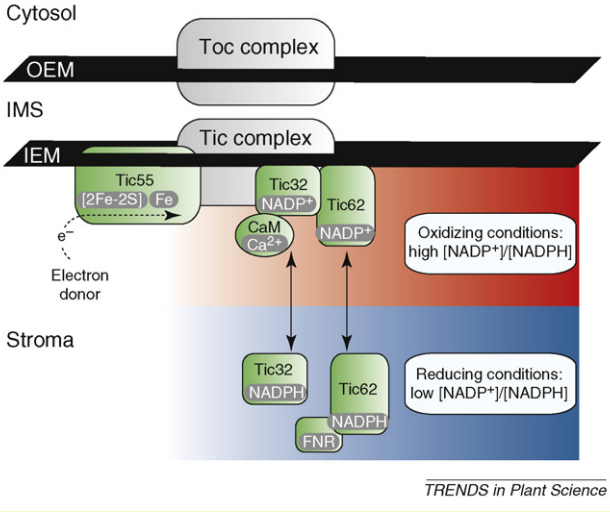


**Figure I.** Tic55, Tic32 and Tic62 as biosensors. The subunit Tic55 is putatively reduced by an electron donor. Pools of Tic32 and Tic62 molecules shuttle between association with the Tic complex (under oxidizing conditions in the plastid) and solubility in the stroma (reducing conditions). The binding of calmodulin (CaM) to Tic32 is stimulated by $Ca^{2+}$ and also causes association of Tic32 to Tic110 [19]. Similarly, ferrodoxin-NAD(P)$^+$ reductase (FNR) is predominantly bound to Tic62 under reducing conditions [20].

and tease out its primitive and derived components and their associated functions.

### The regulatory subunits Tic55, Tic62 and Tic32 might act as metabolic enzymes

Tic55 is proposed to be a regulatory subunit of the Tic complex whose cofactors, [2Fe-2S] and mononuclear iron, act as biosensors (Box 2) [6]. Nonetheless, these cofactors are a common feature observed in aromatic ring-hydroxylating enzymes, which are broadly distributed in prokaryotes [7]. A branch of this family, referred to as lethal leaf spot (Lls), is present in cyanobacteria, algae and plants and includes members such as chlorophyll *a* oxidase (CAO), pheophorbide *a* oxygenase (PAO) and the 52 kDa protein PORA translocation complex (PTC52). Tic55 is also an integrant of the Lls subgroup that is related to the above-mentioned members and particularly closely related to cyanobacterial homologues [7]. This observation indicates that Tic55 might conserve its ancestral prokaryotic properties and that the function of Tic55 merits reinterpretation. It is conceivable that this protein, in addition to its role in protein translocation, might act as a metabolic enzyme (i.e. an oxygenase) [7]. CAO, PAO and PTC52 all have roles in chlorophyll (Chl) metabolism [7–10], therefore by inference Tic55 might also function in Chl metabolism [7]. The degradation pathway of Chl *a* in plants requires hydroxylation of the ethyl chain of pyrrole B by an unknown oxygenase [11]. Curiously, green algae, whose Chl *a* degradation pathway involves fewer enzymatic steps than in plants, have dispensed with this dedicated oxygenase. In algae, the end point of Chl *a* breakdown corresponds to a step of the pathway that in plants occurs before the hydroxylation reaction [11,12]. Using BLAST [13] searches, we only detected the presence of Tic55 in the genome of plants and not in the green algae *Chlamydomonas reinhardtii*, *Volvox carteri*, *Ostreococcus* spp. and *Micromonas* spp., where only homologues with higher similarity to CAO, PAO and PTC52 are present (J. Gross and D. Bhattacharya, unpublished observations; [14]); these findings are consistent with the putative participation of Tic55 in the late hydroxylation step of the Chl *a* breakdown pathway. The Tic55 homologues CAO and PTC52 are reported to interact with protein import sub-complexes involving Toc and Tic components [10,15], suggesting that a link between Chl metabolism and protein import might exist and that Tic55 could potentially play a part in this process.

Tic62 and Tic32 might recapitulate the case of Tic55. Both subunits belong to protein families of NADPH-using dehydrogenases in plants that have a putative ancestral origin [14,16–18]. Tic62 provenance clearly traces back to a cyanobacterial homologue [14,17], whereas Tic32 is related to short-chain dehydrogenase/reductase (SDR) enzymes in prokaryotes and eukaryotes that display a broad range of substrate specificities (Figure 1b) [14,18]. Owing to their membership in protein families with emergent roles in metabolism, it is possible that Tic62 and Tic32 might act as enzymes that have specific metabolic substrates. In fact, for both proteins an enzymatic dehydrogenase activity has been demonstrated *in vitro* [19,20]. The transient nature of the interaction of Tic32 and Tic62 with the Tic translocon further suggests that their regulatory input to the protein import process (Box 2) presumably evolved later and was superposed on the ancient metabolic properties. We suggest that identification of the Tic62 and Tic32 putative substrates might confirm this hypothesis and provide valuable insights into their mode of action in the Tic translocon.

### Tic21 (PIC1): a case study for the evolution of multiple functions

Tic21 was identified as a new member of the Tic complex in a forward genetic screen in *Arabidopsis thaliana* [21]. Its function as a genuine Tic subunit is supported by protein import assays with isolated chloroplasts of *tic21* mutant plants (Box 3) and co-immunoprecipitation experiments demonstrating the physical association of Tic21 with the major Toc and Tic subunits [21]. Surprisingly, plants with genetic defects assigned to *Tic21* in *Arabidopsis* (At2g15290) are impaired in iron homeostasis in the chlor-

**Box 3. How a component of the Toc and Tic translocons is characterized**

In a typical *in vitro* assay, isolated chloroplasts of *P. sativum* uptake exogenous pre-proteins in a three-step reaction dependent on the added amount of nucleotide triphosphate (NTP). In an 'energy-independent' step, pre-proteins make superficial contacts with the Toc receptors in the absence of any NTP [23,35]. An 'early' import stage is reached when addition of ~0.1 mM ATP allows pre-proteins to cross the OEM (i.e. the Toc machinery). If more than 1.0 mM ATP is added to the assay, proteolytic cleavage products appear that correspond to 'late' import substrates that have crossed the IEM (i.e. the Tic translocon) and had their TPs removed by the SPP in the plastid interior. The energy dependence of the assay provides the opportunity to trap translocation intermediates in close association with the Toc machinery (in the 'energy independent' and 'early' stages) or with the Tic complex (in the 'late' translocation step). The arrested pre-proteins serve as a marker for purifying specific translocons using methods involving co-fractionation, in particular epitope-tagged immunoaffinity purification (e.g. [18,23]), blue native PAGE (e.g. [6,16]) and reversible crosslinking (e.g. [23,35]). The protein complexes pulled down by these methods can be resolved by SDS-PAGE, which permits the identification of components by their molecular mass. The further isolation of cDNA clones is possible by screening libraries via degenerate oligonucleotide primers, which are designed based on microsequencing of SDS-PAGE-spotted proteins (e.g. [6,16,18,23,34]). Co-immunoprecipitation is another method that is widely used to provide further evidence that different components interact in the same complex (e.g. [6,16,21,34,35,46]).

The isolation of the *Tic21* gene by a genetic screen in *Arabidopsis* demonstrated a new, powerful tool for identifying components of the Toc and Tic translocons [21]. In addition, *Arabidopsis* (e.g. [24,32,33,36,43,44,48]) and *Physcomitrella patens* (e.g. [47]) lines with specific mutations affecting translocon subunits have been successfully used to further characterize known protein-import components and analyse their function *in vivo*. Protein-import assays are possible with chloroplasts isolated from these *Arabidopsis* mutants, and different nucleotide conditions can be manipulated in the assay to identify the step of the reaction where pre-protein import rates become limited. For example, a protein-import reaction using chloroplasts of the *tic20* [24] and *tic21* [21] *Arabidopsis* knockdown mutants revealed that binding of radioactively labelled pre-proteins to chloroplasts was significantly lower in the mutant than in wild-type organelles only at the 'late' stage of import (i.e. >1.0 mM ATP added). This result provided evidence that Tic20 and Tic21 have a function at the level of the Tic machinery [21,24].

oplast [22]. At2g15290 has a close phylogenetic relationship to cyanobacterial proteins annotated as putative metal ion transporters, resulting in the naming of At2g15290 as PERMEASE IN CHLOROPLASTS 1 (PIC1). Consistent with its new proposed function, *Arabidopsis PIC1* is able to complement (in a heterologous expression system) the yeast double mutant *fet3 fet4*, which is constitutively impaired in iron uptake [22].

We believe that the dual functions of Tic21 as an iron permease and a protein import factor are not in conflict but are instead congruent with the proposed multifunctional properties of Tic55, Tic62 and Tic32 as metabolic enzymes and regulatory subunits of the translocon. In addition, the fact that the Tic21 cyanobacterial homologue Sll1656 was also able to rescue the iron transport deficiency of the *fet3 fet4* yeast double mutant indicates that Tic21 preserves an ancestral pre-endosymbiotic function [22]. We suggest that the case of Tic21 exemplifies what might be considered a hallmark of the Tic subunits: multiple functions reflecting the gain of new properties in protein import that were superposed on the original, ancient function. This hypothesis is used below to revise the models for Tic20 and Tic22 function.

## Revaluating the functions of Tic20 and Tic22

Tic20 is an integral protein in the inner envelope membrane (IEM) of chloroplasts [23]. The function of Tic20 in protein import has been supported by its direct interaction with pre-proteins in crosslinking experiments, its association with other Tic components and specific defects of the *Arabidopsis tic20-I* antisense mutants in protein translocation at the level of the IEM of chloroplasts (Box 3) [23,24]. Recently, a conditional null-mutant of the *tic20* homologue in *Toxoplasma gondii* was shown to be impaired in protein import to the remnant apicomplexan plastid (apicoplast) [25]. A mechanistic function for Tic20 was originally proposed based on a PSI-BLAST search that retrieved similarities of Tic20 with branched amino acid transporters, a family of proteins considered to be homologues of Tim23 and Tim17 (subunits of the translocase of the mitochondrial inner membrane) [4]. By inference, it was proposed that Tic20 is also a Tim23 and Tim17 homologue that comprises an analogous protein-conducting channel at the IEM of chloroplasts [4]. We repeated the PSI-BLAST [13] search using the *Pisum sativum* Tic20 protein (accession number AAC64607) and the *Synechocystis* sp. PCC 6803 homologue (NP_440747) as queries. Surprisingly, we were unable to reproduce the previous hits with branched amino acid transporters or with any protein with a putative function in peptide translocation (results not shown). This suggests that the previous inclusion of Tic20 in the broad family of amino acid transporters [4] might have been an artefact of the size of the sequence database at that time, and therefore evidence favouring Tic20 as a protein channel should be re-visited.

In this regard, no direct evidence has thus far been provided in support of the mechanistic function of Tic20 as a protein-conducting channel of the Tic translocon. A *T. gondii tic20* mutant that was impaired in protein import into the apicoplast still functioned at 65%–77% of wild-type levels when the accumulation of Tic20 protein in the conditional null-mutant was below the limit of detection [25]. This result is incompatible with the idea that Tic20 is the IEM entrance channel for proteins targeted to the interior of the apicoplast [25]. In addition, the observed low expression of *Arabidopsis tic20-I* and *tic20-IV* isoforms at both the level of RNA [21,26] and protein [26,27] suggests that Tic20 is sub-stoichiometric to other Tic components and Toc75 [21,26,27]. This also speaks against the role of Tic20 as a general protein import pore at the IEM of chloroplasts [26]. However, it has been recently proposed that during plant development, *Arabidopsis* Tic21 might have a pattern of expression complementary to Tic20 and might form a channel for the Tic complex that compensates for the low accumulation of Tic20 [21]. It is not clear whether this putative Tic channel is a combination of both Tic21 and Tic20 or is constituted by Tic21 alone [21].

Despite its still uncertain role in protein translocation, Tic20 recapitulates the case where a cyanobacterial-derived protein (Figure 1b) is phylogenetically conserved across a taxonomically broad array of photosynthetic eukaryotes [4,5,14]. As we hypothesized above, this might imply preservation of ancient pre-endosymbiotic traits. An elusive protein import-related anion channel (PIRAC) has been electrophysiologically assigned to the IEM of chloroplasts, and the channel's activity is modulated by pre-proteins that interact directly with the Tic translocon and/or PIRAC and inhibited by the addition of specific antibodies raised against Tic110 [28,29]. Moreover, blockage of PIRAC by the anion channel inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonate reduces pre-protein import efficiency in a dosage-dependent manner [29]. The functional association of PIRAC with the Tic translocon, its IEM localization and its interaction with pre-proteins and Tic110 are reminiscent of the characteristics observed for Tic20 [23,24]. Assuming that functional duality is a hallmark of the Tic translocon, it is tempting to speculate that Tic20 and PIRAC are the same protein. In accordance with a putative anionic selectivity, Tic20 has a high concentration of positively charged amino acids that are putatively exposed to the intermembrane space (IMS) and stroma [23].

Tic22 is another Tic-complex component [23] whose function in protein import is supported by association of the *P. sativum* Tic20 with other translocon components and pre-proteins during translocation. However, the proposed role of Tic22 as a connector between the Toc and Tic translocons has never been directly tested. Nonetheless, Tic22 is a member of a plastid-localized protein family of cyanobacterial origin [4,5,14] (Figure 1b) that is present in all available algal genomes with the exception of the green picoeukaryotic algae *Ostreococcus* spp. and *Micromonas* spp. (J. Gross and D. Bhattacharya, unpublished observations; [14]). We again propose that the conservation of Tic22 reflects an ancestral prokaryotic property that has been broadly maintained alongside its function in protein import. In accordance with this idea, the Tic22 homologue in *Synechocystis* sp. PCC 6803 (Slr0924) is essential for cell viability [30]. Its accumulation and probably its function seem to be controlled by the redox status of photosynthetic electron flow [30].

### Tic110 and Tic40 might be the only Tic components with an exclusive function in protein import

Tic110 is a key component of the Tic translocon [31–33]. Its essential nature is clearly illustrated by the embryo-lethal phenotype resulting from the *Arabidopsis tic110* knockout and is in accordance with a central function of Tic110 in the Tic translocon [32,33]. In addition, Tic110 constitutes the Tic-complex backbone, which directly interacts with the Toc complex and all other Tic components [6,16,18,21,23,33–35], as well as with the stromal ClpC (caseinolytic protease subunit C), a heat shock protein 93 kDa (Hsp93) that is the driving force for the translocation process across the IEM [35–37]. The idea that Tic110 might be a protein-conducting pore of the complex is supported empirically by the reconstitution of the recombinant *P. sativum* Tic110 in planar lipid bilayers as a cation-selective and high-conductance channel presenting affinity for TP-containing proteins [31]. The expression of *Arabidopsis* Tic110 in different tissues and stages of plant development with an accumulation pattern comparable to the one observed for Toc75 [26,33] is also in agreement with a potential function of Tic110 as a protein pore at the IEM of chloroplasts. In contrast with the other Tic subunits discussed above, Tic110 has homologues only in the genomes of Plantae (Figure 1b) [4,5,14], indicating that this protein was generated *de novo* in the host, presumably to assume an exclusive function in protein translocation (Figure 1b). Due to its essential nature, its central position in the translocation process and its apparent lack of dual functions, we suggest that Tic110 was a crucial component of the ancestral Plantae translocon, acting at the same time as the primitive protein-conducting pore at the IEM and as a module to recruit the associated ClpC chaperone (Figure 1c).

However, this simplified view of Tic110 as the sole protein-conducting channel of the Tic translocon still awaits conclusive experimental evidence. The original *in vitro* reconstitution experiments with *P. sativum* Tic110 predicted it as a protein channel with a structure predominantly composed of β-sheets [31]. However, the results of this study were obtained with urea-denatured Tic110 recombinant protein, whereas the purified native protein could not be reconstituted in planar lipidic bilayers [31]. In addition, the 97.5 kDa C-terminal domain of *Arabidopsis* Tic110 was purified as a soluble protein containing a high proportion of α-helices [38], suggesting that the C-terminal portion of the protein might not form a membrane pore but rather a soluble module protruding into the stroma [38].

In contrast to Tic110, Tic40 seems to be an auxiliary factor for ClpC function [32,34,36,37], suggesting that this component is not essential for protein translocation. This idea is largely corroborated by the viable phenotype of the *tic40* T-DNA knockout in *Arabidopsis* [32,36]. For this reason, it is more likely that Tic40 was a later addition to the primitive 'core translocon' (Figure 1c).

### The function of most Toc subunits seems to be specific to protein translocation

In contrast to the Tic complex (see below), the Toc complex can be reconstituted *in vitro* and purified with a defined stoichiometric subunit composition [39,40]. In addition, a mechanistic role in the process of protein import can be unequivocally assigned for the majority of the Toc subunits. Toc75 is the most prominent subunit of the complex, which serves as a protein-conducting channel for the translocon [41,42]. In accordance with this essential function, *Arabidopsis* plants containing a T-DNA insertion in the Toc75 encoding gene (*AtToc75-III*) have an embryo-arrested phenotype [43,44]. Furthermore, *AtToc75-III* is expressed throughout plant development in different tissues and is abundant in young, rapidly dividing photosynthetic cells where demand for protein import is higher [26,43]. The essential function of Toc75 in modern-day plastids speaks in favour of the idea that this component was a core constituent of the ancestral Plantae translocon (Figure 1c).

Toc34 is probably derived from an ancient GTPase of eukaryotic provenance (Figure 1b) [5], whereas Toc159 is believed to have originated from Toc34, possibly via gene duplication and tandem fusions of its ancient GTPase domain [5,45]. The essential need for a pre-protein receptor at the cytosolic surface of the organelle implies that Toc34 in its ancestral form might have been a putative component of the primitive translocon (Figure 1c). Toc12 has a co-chaperone function and is usually described as an auxiliary factor [46], whereas Toc64 seems to be 'dispensable' because double and triple *toc64*-knockout lines of *Physcomitrella patens* and *Arabidopsis*, respectively, are not defective in protein import and seem to be devoid of any apparent abnormal phenotype [47,48]. Toc12 and Toc64 might have been added later to the translocon during evolution of the green lineage.

### From the ancestral Plantae translocon towards the complex machinery in plants

A reductive analysis of Toc and Tic functions leads to specific conclusions about plastid translocon evolution. We propose that the core of the primitive translocon was composed of the Toc75 and Tic110 pores and the Toc34 receptor (Figure 1c). We suggest that the inclusion of Tic20 and Tic21 as constituents of the ancestral IEM translocon (Figure 1c) still awaits direct experimental evidence for their function as protein-conducting pores (e.g. reconstitution and eletrophysiological characterization of the protein channels in lipid membrane systems). The molecular chaperones' essential function of keeping pre-proteins in an import-competent unfolded state and providing the motor force for translocation [37,49] implies that the associated chaperones Hsp70 and ClpC were engaged early on in protein translocation (Figure 1c). The remaining subunits are later additions to the translocon: most of them accrued in the green lineage via co-option of existing proteins, and in some cases the original functions of the subunits were retained.

We propose that the auxiliary factors Toc12 (which is related to DnaJ-type Hsp70 co-chaperones of uncertain phylogenetic provenance [5,14]) and Tic40 (which is derived from the eukaryotic co-chaperones Hip [Hsp70-interacting protein] and Hop [Hsp70–Hsp90 organizing protein] [36,37]) were recruited to assume a new exclusive function in protein translocation (Figure 1c). Functional specialization is more obvious for Toc159, which originated

via duplication of Toc34 to increase or modulate the specificity of pre-protein recognition by the Toc translocon [5,14,45]. These two receptors underwent new rounds of duplications in plants where the isoforms Toc34/Toc33 and Toc159/Toc132/Toc120/Toc90 are encoded in these genomes and believed to be engaged in the formation of alternative translocons [5,14].

We suggest that the predominant trend in evolution towards translocon complexity involved the recruitment to the Tic complex of subunits that assumed a new function in protein import but that at the same time retained their original pre-endosymbiotic properties (Figure 1c). The resulting functional superposition might explain the duality prevalent among Tic subunits. Under this scenario, it is possible that a partitioning of subunit pools exists that accounts for the preferential association or disassociation with the Tic complex. It is conceivable that subunits that are disassociated from the Tic complex are predominantly engaged in ancestral functions, whereas a specific subgroup that interacts with Tic110 switches to the protein translocation mode. The existence of flexible pools of subunits might explain the observation in *P. sativum* that the bulk of Tic22, Tic20 and Tic110 molecules do not conjunctly associate within a complex [23]. The lack of a defined stoichiometry between Tic components has been suggested by different studies [21,23,26,27,50].

Another intriguing possibility is that the multifunctionality of Tic110 interactors might have a purely informational nature, suggesting that Tic components act as 'expanded' regulatory subunits. This implies that Tic components not only relay redox and calcium signals to the translocon (Box 2) but also transmit the status of the different conditions for each biological process associated with a particular Tic subunit (e.g. metabolism, Chl breakdown, ion permeation) to the Tic channel to regulate its protein-import activity. This idea that the Tic translocon is a central point for regulatory networks within the plastid is in line with the proposed modulatory function of Tic40 [50,51]. During plant development, the accumulation of this subunit fluctuates in comparison to the constitutive expression of Tic110, a fact that might have implications for the regulation of protein import activity [50]. In addition, recent evidence suggests that Tic40 might be a target of rhomboid proteases, which are key players in the signal transduction pathway of regulated intra-membrane proteolysis [51].

Beyond the notion that Tic40 and other Tic110 interactors have expanded modulatory properties is the reciprocal possibility that the status of protein import through the putative Tic110 channel might itself modulate different metabolic and physiological processes represented by the Tic110 interactors (e.g. metabolic flow rates, solute transport activities). Under this scenario, the Tic translocon would act as a prominent macro-regulator of broad plastid functions, interconnecting and co-adjusting different organelle processes. This proposed role for the multifunctional protein aggregate in systemic regulation might transcend the notion of the Tic complex as conventional translocator machinery: perhaps it is better described as a 'molecular integrator'. This paradigm for the Tic translocon recapitulates the multifunctional nature of the nuclear pore complex, which is known to recruit and regulate the activity of gene expression, RNA processing and export machineries along the nuclear envelope [52]. This observation suggests that trafficking points at the boundaries of eukaryotic intracellular compartments evolve into molecular hubs that interconnect and modulate different vital cellular activities.

## Concluding remarks

Our hypothesis of functional duality as a key attribute of some plastid translocon subunits provides not only the opportunity to re-think its properties but also to design future experiments to assess Toc and Tic subunit functions. This is particularly true for genetic studies that often uncover dramatic phenotypes associated with Toc and Tic mutants (e.g. *Arabidopsis* Tic20 [24] and Tic21 [21] and apicomplexan Tic20 [25]). It is possible that these phenotypes not only indicate the impairment of protein import but also reflect broader pleiotropic effects arising from general loss of plastid functions. We propose that the concept of functional duality should be addressed in future studies of the Toc and Tic subunits by experiments aimed at testing these proteins for alternative properties. The analysis of PIC1 in *Arabidopsis* as an iron transporter in chloroplasts is an example of such a strategy [22]. This approach might be useful for testing the Tic 'molecular integrator' hypothesis, as well as the general validity of our speculations that subunits with dual functions were late additions to the ancestral translocon and tend not to be essential for protein translocation.

## Note added in proof

While correcting the final proof of this manuscript, we became aware of a recent report that demonstrates the reconstitution of both native and recombinant forms of Tic110 in planar lipid bilayers as cation-selective pores [53]. Topological studies suggest that Tic110 has a total of six transmembrane helices. Four of them are putatively located in the C-terminal region and might be involved in the constitution of the protein pore. This study provides an answer to objections previously raised in the literature about the function of Tic110 as a protein pore (e.g. [38]). In addition, it provides solid support for our model that Tic110 presumably was the sole constituent of the primitive protein-conducting channel in the IEM of the ancestral plastid.

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