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REVIEW

Evolution of protein transport to the chloroplast envelope membranes

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Abstract
Chloroplasts are descendants of an ancient endosymbiotic cyanobacterium that lived inside a eukaryotic cell. They inherited the prokaryotic double membrane envelope from cyanobacteria. This envelope contains prokaryotic protein sorting machineries including a Sec translocase and relatives of the central component of the bacterial outer membrane β-barrel assembly module. As the endosymbiont was integrated with the rest of the cell, the synthesis of most of its proteins shifted from the stroma to the host cytosol. This included nearly all the envelope proteins identified so far. Consequently, the overall biogenesis of the chloroplast envelope must be distinct from cyanobacteria. Envelope proteins initially approach their functional locations from the exterior rather than the interior. In many cases, they have been shown to use components of the general import pathway that also serves the stroma and thylakoids. If the ancient prokaryotic protein sorting machineries are still used for chloroplast envelope proteins, their activities must have been modified or combined with the general import pathway. In this review, we analyze the current knowledge pertaining to chloroplast envelope biogenesis and compare this to bacteria.

Keywords Chloroplast · Endosymbiosis · Envelope · Protein sorting · Transmembrane

Introduction

Chloroplasts are the result of an ancient endosymbiotic relationship between a species of cyanobacteria and the eukaryotic ancestor of land plants, green algae, red algae, and glaucophytes. Through this endosymbiosis, photosynthesis via the z-scheme and the Calvin–Benson–Bassham cycle were passed from prokaryote to eukaryote. Over the course of evolution, structural similarities between chloroplasts and cyanobacteria remained. While the most obvious of these are the thylakoid membranes, the respective envelope membranes also retain bacterial properties. Cyanobacterial cell envelopes are composed of two membranes with the periplasm and peptidoglycan cell wall between them (Hoiczyk and Hansel 2000). Chloroplasts from all organisms in the Archaeplastida clade retain the double membrane and some retain the peptidoglycan wall (Hirano et al. 2016; Inoue 2011). Although these structures are conserved, many aspects of their biogenesis are not since nearly all the genes coding for proteins in these compartments exist in the nuclear genome. These genes are translated in the eukaryotic cytosol, meaning the initial direction the proteins approach their final location is the opposite of cyanobacteria. Here, we review the mechanisms that plant cells evolved to allow this change in direction.

The chloroplast envelope membranes are necessary to maintain the stroma as a distinct cellular subcompartment. As the barrier between the chloroplast and the rest of the cell, the envelope contains many solute transport proteins necessary to maintain metabolic connection to the rest of the cell. Most of the characterized transporters are found in the inner membrane and it is believed to be the major site of transport regulation (Facchinelli and Weber 2011). The outer membrane (OM) has long been considered non-selectively permeable to small solutes, but this idea is debated (Harsman et al. 2016; Inoue 2011). Both envelope membranes are important for lipid synthesis and trafficking (Hurlock et al. 2014). The envelope also houses proteins involved in chloroplast division (Osteryoung and Pyke 2014). The evolution of targeting mechanisms to sort cytosolically translated proteins to the chloroplast envelope allowed both the migration of endosymbiont genes to the host nucleus as well as the introduction of novel proteins. This was important for chloroplast evolution because the essential processes that

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occur at the chloroplast envelope are carried out by proteins of both cyanobacterial and eukaryotic origin.

Most of the proteins in the stroma and thylakoid lumen, and many in the thylakoid membrane, are coded for by nuclear genes (Celedon and Cline 2013). They are translated as precursors with N-terminal transit peptides that are both necessary and sufficient for chloroplast targeting and import (Lee and Hwang 2018). The precursors pass through the envelope through the translocons at the outer and inner envelope membranes of chloroplasts (TOC/TIC). The TOC complex contains Toc75, Toc159, and Toc34 (Kessler et al. 1994; Schnell et al. 1994). Toc75 is thought to function as the import pore. It evolved from a cyanobacterial protein that probably functioned in OM protein insertion (Bolter et al. 1998; Inoue and Potter 2004; Reumann et al. 1999). Toc159 and Toc34 are GTPase receptors that appeared after endosymbiosis (Reumann et al. 2005). Tic22 is thought to link the TOC and TIC and likely has chaperone activity (Glaser et al. 2012; Kouranov et al. 1998). Although it is named Tic22, it is in the IMS and interacts directly with Toc75 (Paila et al. 2016). The cyanobacterial homolog of Tic22 is important for OM biogenesis (Tripp et al. 2012). Many other potential TIC components have been identified including Tic20, Tic40, Tic110, Tic21, Tic214, Tic100, Tic55, Tic56, Tic32, and Tic62 (Bolter and Soll 2016). Whether these are truly part of the TIC and what roles they may play are not known. After exiting the TIC and entering the stroma, the transit peptide is removed by the stromal processing peptidase (VanderVere et al. 1995).

Proteins destined for the thylakoids generally contain additional targeting information following the transit peptide in the form of a signal peptide or transmembrane domain. These regions direct precursors to the cpSRP, cpSec1, and Twin arginine translocase (Tat) pathways (Celedon and Cline 2013).

The targeting machinery shared by both stromal and thylakoid proteins is referred to as the general import pathway. If proteins destined for the envelope compartments use components of this general import pathway, they must have mechanisms to either prevent full translocation into the stroma or be exported back to the envelope from the stroma. Examples of inner membrane proteins using each of these methods have been identified. Less is known about protein targeting to the OM and intermembrane space (IMS), but several of these proteins have also been shown to use components of the general import pathway.

Inner envelope membrane

The chloroplast IM is homologous to the bacterial plasma membrane. In proteobacteria, plasma membrane proteins are inserted co-translationally by the signal recognition particle (SRP) pathway or post-translationally by the Sec translocase. Both of these use the SecYEG translocon (Kuhn et al. 2017). Although little research has been carried out directly on protein insertion into the cyanobacterial plasma membrane, it contains most of the components of the SRP pathway and Sec translocase (Frain et al. 2016). Cyanobacterial IM proteins likely use one or both of these pathways (Fig. 1a).

Nearly all the chloroplast IM proteins are translated in the eukaryotic cytosol and thus start out separated from their final location by the OM and in some cases

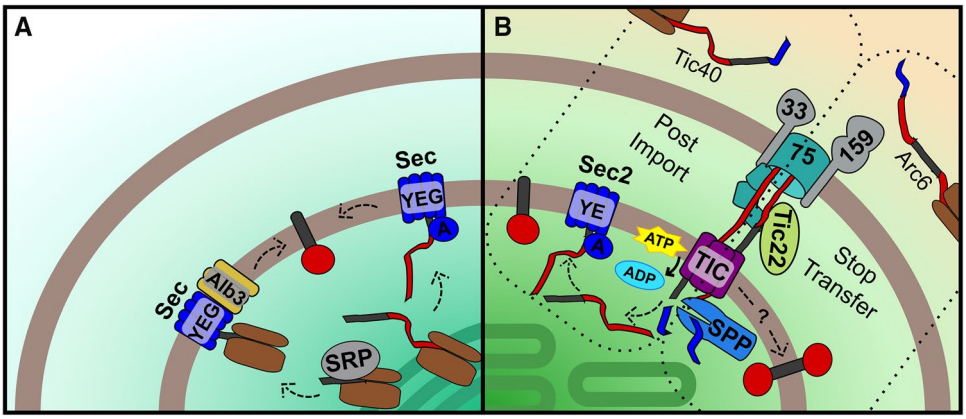


Fig. 1 Models of inner membrane protein targeting and insertion. **a** Cyanobacteria contain the necessary components for both co-translational insertion by the SRP pathway and post-translational insertion by the Sec translocase. Both pathways use the SecYEG translocon. **b** Chloroplast inner membrane proteins are translated in the eukaryotic cytosol meaning they must be post-translationally sorted to the chloroplast before insertion into the inner membrane. An N-terminal transit peptide (blue) targets them for import through the translocons at the outer and inner envelope membranes of chloroplasts (TOC/TIC) and is removed by the stromal processing peptidase (SPP). Two pathways have been identified for sorting to the inner membrane. Substrates of the post-import pathway, represented by Tic40, are fully imported into the stroma before insertion via an envelope-localized Sec translocase. Substrates of the stop-transfer pathway, represented by Arc6, stall in the translocon and are laterally released into the membrane

a peptidoglycan wall. This means that co-translational insertion into the IM is not possible even though chloroplasts retain protein components of the SRP pathway. An exception to this may be the recently identified Tic214 which is the only known integral IM protein encoded by a gene in the chloroplast genome (Kikuchi et al. 2013). Of the IM proteins that are post-translationally imported into chloroplasts, two mechanisms of IM integration have been observed consistently, referred to as the stop-transfer and post-import pathways (Fig. 1b). In both cases, IM proteins are translated with transit peptides that direct them to the TOC and TIC, and information near or within the transmembrane domain sorts them to the IM (Knight and Gray 1995; Singhal and Fernandez 2017; Tripp et al. 2007; Viana et al. 2010). The pathways are distinguished by the absence or presence of a soluble intermediate seen during in vitro import assays, respectively.

The first IM protein targeting pathway identified was stop transfer (Li et al. 1992). In this pathway, a hydrophobic region in the mature protein is responsible for envelope sorting (Brink et al. 1995). Further, the transmembrane domain from the stop transfer substrate Arc6, a component of the chloroplast division apparatuses, was sufficient to redirect transmembrane thylakoid proteins to the IM (Froehlich and Keegstra 2011; Tripp et al. 2007). In this pathway, it is thought that the hydrophobic region prevents full translocation into the stroma by stalling in the TIC channel and being laterally released into the IM (Firlej-Kwoka et al. 2008). Direct release from the TIC to the membrane has not been experimentally shown, so it remains possible that the proteins are passed to another protein complex during insertion.

The post-import pathway was initially distinguished from the stop transfer pathway by the appearance of soluble intermediates of Tic110 in in vitro chloroplast import assays (Lubeck et al. 1997). A similar intermediate appears for Tic40, and this soluble, processed protein could be inserted into isolated IM vesicles (Li and Schnell 2006). The post-import pathway is also referred to as the conservative sorting pathway as it more closely resembles protein sorting in bacteria by restoring the direction the protein approaches the membrane and may require conserved translocon components at the IM. Components of the Sec translocon have long been known to exist in the thylakoid (Nakai et al. 1994; Yuan et al. 1994). More recently, an envelope-specific Sec (Sec 2) was identified (Li et al. 2015; Skalitzky et al. 2011; Fernandez 2018). Also, plastidic type I signal peptidase 1 (Plsp1), which is dual localized to the thylakoid and IM, was shown to use Sec components for thylakoid insertion, suggesting it may also use Sec 2 components at the envelope (Endow et al. 2015; Klasek and Inoue 2016). Recently, direct evidence for the involvement of Sec 2 components in the insertion of Tic40 and the protease FTSH12 was obtained (Li et al. 2017).

The role of envelope-localized Sec in the sorting of IM proteins suggests that, in terms of proteins responsible, the post-import pathway really is conservative. However, in terms of substrates, the post-import pathway is not conservative since Tic40 and Tic110 appeared after endosymbiosis (Reumann et al. 2005; Reumann and Keegstra 1999; Shi and Theg 2013). In contrast, the stop transfer substrate Arc6 evolved from a component of the cyanobacterial division machinery, and its cyanobacterial ancestor presumably used the Sec channel to insert into the IM (Vitha et al. 2003). If the TIC ejects stop transfer substrates laterally into the IM, this means that over the course of evolution Arc6 and other proteins adopted a new strategy for membrane insertion. How this mechanism evolved is not known but is likely closely tied to the evolution of the TIC complex. Several IM proteins whose targeting has been investigated are included in Table 1. Most are classified as stop transfer simply due to the lack of any observed soluble intermediates during chloroplast import. These include proteins of both cyanobacterial and eukaryotic origin. They have three possible membrane topologies including C-in, N-in, and polytopic. Polytopic IM proteins may use both lateral release from the TIC and Sec 2 for membrane insertion, as has been suggested by Singhal and Fernandez (2017).

Intermembrane space

The IMS of chloroplasts is homologous to the periplasm of bacteria. In bacteria, proteins are targeted to this compartment post-translationally by a signal peptide which sends the precursor protein to either the Sec or the Tat translocases. The signal peptides are removed in the periplasm by a membrane-bound peptidase (Fig. 2a). Although a Sec translocase and a signal peptidase exist at the chloroplast inner membrane (Inoue et al. 2005; Li et al. 2015), IMS proteins may not use these components since they only need to pass the OM to reach their functional location.

The targeting of only two IMS proteins has been investigated in detail. Tic22 is a component of the protein import apparatus that bridges the TOC and TIC complexes (Kouranov et al. 1998; Paila et al. 2016). It is translated in the cytosol with a presequence that is similar to canonical transit peptides but takes its passenger to the IMS rather than the stroma (Kouranov et al. 1999). Import of Tic22 requires proteinaceous components at the OM, probably the TOC complex (Kouranov et al. 1998; Vojta et al. 2007). Interestingly, the presequence of Tic22 was not removed by isolated stroma, suggesting that it may be processed by a peptidase other than the stromal processing peptidase (Vojta et al. 2007). Together these results allow for the possibility that Tic22 passes through only the TOC translocon and directly enters the IMS without engaging the TIC machinery (Fig. 2b). The import of Tic22 is enhanced by ATP

Table 1 Shows a collection of chloroplast envelope proteins

AGI no.	Name	Transit peptide?	Toc and/or Tic?	Location	Envelope sorting mechanism	Membrane topology	Origin	References
At5g16620	Tic40	Yes	Yes	IM	Post-import/ conservative (Sec2)	C-in α-helix	Eukaryotic	Li and Schnell (2006), Viana et al. (2010), Li et al. (2017), Reumann et al. (2005)
At1g06950	Tic110	Yes	Yes	IM	Post-import/ conservative	Polytopic	Eukaryotic	Lubeck et al. (1997), Reumann et al. (2005)
At5g42480	Arc6	Yes	Yes	IM	Stop-transfer	N-in α-helix	Cyanobacterial	Vitha et al. (2003), Tripp et al. (2007), Froehlich and Keegstra (2011)
AtCg01130	Tic214	NA	NA	IM		Polytopic		Kikuchi et al. (2013)
At1g79560	FtsH12	Yes	Yes	IM	Sec2	Polytopic	Cyanobacterial	Nishimura et al. (2016), Li et al. (2017)
At1g29395	Cor413im1	Yes	Yes	IM	Stop-transfer	Polytopic		Okawa et al. (2014)
At2g31530	SCY2	Yes	Yes	IM	Stop-transfer	Polytopic	Cyanobacterial	Singhal and Fernandez (2017)
At5g46110	TPT	Yes	Yes	IM	Stop-transfer	Polytopic	Eukaryotic	Brink et al. (1995), Tyra et al. (2007)
At5g33320	PPT	Yes	Yes	IM	Stop-transfer	Polytopic	Eukaryotic	Firlej-Kwoka et al. (2008), Tyra et al. (2007)
At2g15290	Tic21/PIC1	Yes	Yes	IM	Stop-transfer	Polytopic	Cyanobacterial	Firlej-Kwoka et al. (2008), Shi and Theg (2013)
At3g63410	VTE3/APG1	Yes	Yes	IM	Stop-transfer	C-in α-helix		Firlej-Kwoka et al. (2008), Viana et al. (2010)
At2g26900	BASS2	Yes	Yes	IM	Stop-transfer	Polytopic	Eukaryotic	Firlej-Kwoka et al. (2008), Tyra et al. (2007)
At3g51140	HP28	Yes	Yes	IM	Stop-transfer	Polytopic	Cyanobacterial	Firlej-Kwoka et al. (2008), Tyra et al. (2007)
At4g32400	BT1	Yes	Yes	IM	Stop-transfer	Polytopic	Eukaryotic	Li et al. (1992), Kirchberger et al. (2008), Tyra et al. (2007)

Table 1 (continued)

AGI no.	Name	Transit peptide?	Toc and/or Tic?	Location	Envelope sorting mechanism	Membrane topology	Origin	References
At3g24590	Plsp1	Yes	Yes	IM and Thylakoid	Possibly post import (Sec2)	N-in α-helix	Cyanobacterial	Endow et al. (2015)
At4g33350; At3g23710	Tic22	Yes	Debated	IMS	Transit peptide is sufficient	Peripheral	Cyanobacterial	Kouranov et al. (1999), Vojta et al. (2007)
At4g31780	MGD1	Yes	Yes	IMS		Peripheral	Chloroflexi	Miege et al. (1999), Vojta et al. (2007), Yuzawa et al. (2012)
At3g46740	Toc75-III	Yes	Yes	OM	Stop transfer via polyglycine stretch	β-Barrel	Cyanobacterial	Tranel et al. (1995), Tranel and Keegstra (1996), Inoue and Keegstra (2003), Endow et al. (2016)
At4g09080	Toc75-IV	No		OM		β-Barrel	Cyanobacterial	Baldwin et al. (2005)
At5g19620	OEP80	Yes		OM		β-Barrel	Cyanobacterial	Hsu et al. (2012), Day et al. (2014), Day unpublished
At3g44169	OEP80tr/P39	No		OM		β-Barrel	Cyanobacterial	Day et al. (2014), Hsueh et al. (2014)
At2g43950	OEP37	No		OM		β-Barrel		Goetze et al. (2006)
At1g45170; At5g42960	OEP24	No		OM		β-Barrel		Pohlmeyer et al. (1998)
At1g20816; At1g76405	OEP21	No		OM		β-Barrel		Bölter et al. (1999)
At3g57990	OEP40	No		OM		β-Barrel		Harsman et al. (2016)
At3g06960	TGD4	No		OM		β-Barrel	Cyanobacterial	Wang et al. (2012)
At3g52420	OEP14/OEP7	No	Yes	OM	AKR2A	N-in α-helix		Lee et al. (2001), Tu et al. (2004), Bae et al. (2008)
At3g06510	SFR2	No		OM		N-in α-helix		Roston et al. (2014)
At5g05000	Toc34	No		OM	AKR2A	C-in α-helix	Eukaryotic	Bae et al. (2008), Dhanoa et al. (2010), Reumann et al. (2005)
At4g02510	Toc159	No		OM	Interaction with Toc34 and Toc75	C-in unknown structure	Eukaryotic	Wallas et al. (2003), Reumann et al. (2005)

Table 1 (continued)

AGI no.	Name	Transit peptide?	Toc and/or Tic?	Location	Envelope sorting mechanism	Membrane topology	Origin	References
At3g11670	DGD1	No	Yes	OM		N-in α-helix		Hofmann and Theg (2005b)
At3g17970	OEP64	No	Yes	OM	AKR2A	N-in α-helix	Eukaryotic	Hofmann and Theg (2005b), Bae et al. (2008), Reumann et al. (2005)
At1g16000	OEP9	No		OM	AKR2A	C-in α-helix		Dhanoa et al. (2010)

The list is not exhaustive, instead including only proteins whose targeting has been investigated. Blank cells indicated that the information is not known

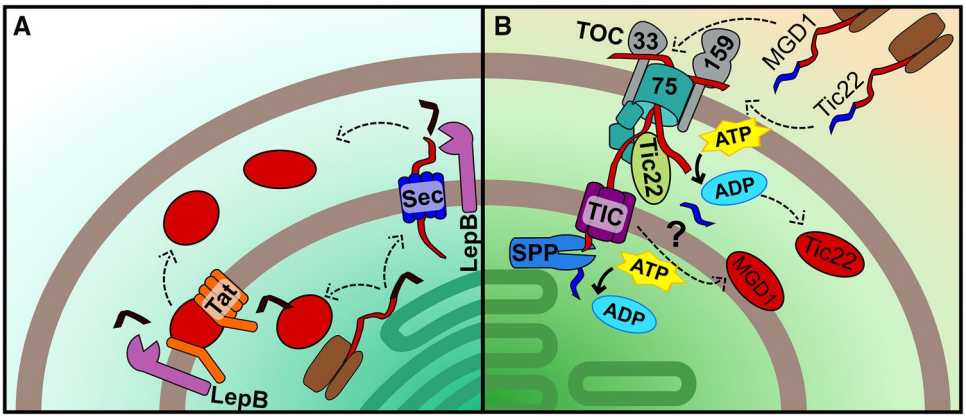


Fig. 2 Models of periplasm/IMS protein targeting. **a** Cyanobacterial periplasmic proteins are translated in the cytosol with a signal peptide. They are exported across the inner membrane through either the twin arginine translocon (Tat) or Sec pathway. The signal peptide is removed by the signal peptidase LepB. **b** Chloroplast IMS proteins are translated in the eukaryotic cytosol with transit peptides (blue) and must pass the OM through the translocon at the outer envelope membrane of chloroplasts (TOC). Tic22 may avoid the translocon at the inner envelope membrane of chloroplasts (TIC) and directly enter the IMS. If this is the case, ATP hydrolysis and removal of the transit peptide would occur in the IMS by unknown factors. MGDG1 at least partially passes through the TIC to have its transit peptide removed by the stromal processing peptidase (SPP)

(Kouranov et al. 1999; Vojta et al. 2007). If Tic22 does pass only though the OM, this means that ATP hydrolysis occurs in the IMS or cytosol.

The other IMS localized protein whose targeting has been studied is MGDG synthase (MGD1). MGD1 associates with the outer leaflet of the IM via amphipathic helices but does not contain a membrane spanning domain (Miege et al. 1999). Like Tic22, MGD1 is translated in the cytosol with a presequence that directs it to the chloroplast (Miege et al. 1999; Vojta et al. 2007). Unlike Tic22, the presequence is removed by isolated stroma (Vojta et al. 2007). This suggests that it is more like canonical transit peptides and that at least part of the MGD1 precursor passes through the TIC apparatus to the stroma. It is not clear if the entire protein enters the stroma and is subsequently exported to the IMS, or if it is prevented from fully crossing the IM and left in the IMS (Fig. 2b).

Outer envelope membrane

In the OM of Gram-negative bacteria, β-barrels are the predominant class of transmembrane protein. These OM proteins (OMPs) are translated in the cytosol with an N-terminal signal peptide, then exported to the periplasm through the Sec translocase. In *E. coli*, the periplasmic chaperones Skp and SurA have been implicated in carrying OMPs to the OM (Rollauer et al. 2015). Once at the OM, OMPs are folded and inserted by the β-barrel assembly module (BAM) containing subunits A through E. BamA contains

a transmembrane β-barrel while BamC–E are lipoproteins (Rollauer et al. 2015) (Fig. 3c).

Although most of these β-barrel sorting components are conserved in proteobacteria, other than the Sec translocase only BamA has homologs in cyanobacteria (Webb et al. 2012). BamA is a member of a large family of proteins called Omp85 homologs found throughout Gram-negative bacteria. These are composed of a C-terminal 16 β-strand barrel and a variable number of N-terminal polypeptide transport-associated domains (POTRA) located in the periplasm. Due to their conservation, Omp85 homologs are thought to be necessary for the folding and insertion of transmembrane β-barrels in all Gram-negative bacteria. The chloroplast outer envelope membrane also contains β-barrels, including Omp85 homologs. All green algae and land plants contain two groups of Omp85 homologs, Toc75, and OEP80. Toc75 is a component of the chloroplast protein import apparatus. The function of OEP80 has not been experimentally verified, but it is hypothesized to retain the ancestral Omp85 function in β-barrel assembly.

Escherichia coli has an additional Omp85 containing complex, called the translocation and assembly module (Tam), that is responsible for the assembly of a subset of β-barrel proteins that include the autotransporters (Selkrig et al. 2012) (Fig. 3c). Autotransporters are a subclass of OM proteins that consist of a transmembrane β-barrel and

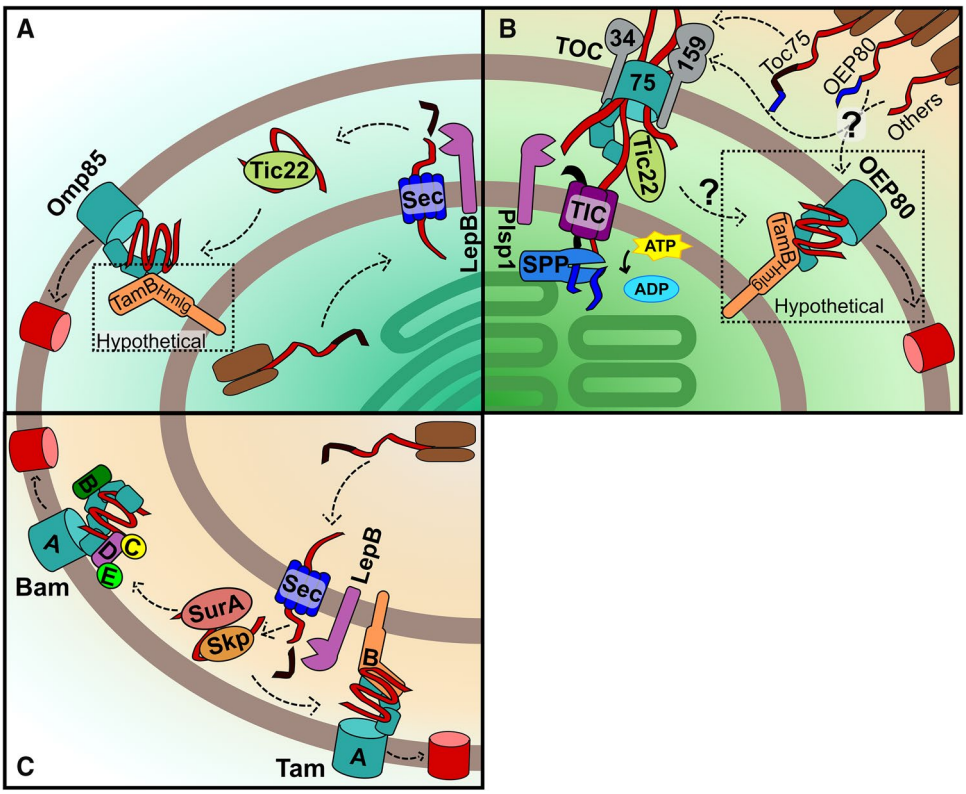


Fig. 3 Models of β-barrel sorting to the OMs of bacteria and chloroplasts. **a** Cyanobacterial OM β-barrels are translated in the cytosol with signal peptides that direct them to the Sec for post-translational export. In the periplasm, they may interact with a cyanobacterial homolog of Tic22. The nascent β-barrels are then inserted into the OM by an Omp85 homolog. *TamB* homologs exist in cyanobacterial genomes and may play a part in β-barrel assembly. **b** Chloroplast β-barrels are translated in the eukaryotic cytosol. In some cases, they are targeted to the chloroplast with transit peptides (blue) (Toc75 and probably OEP80). In most cases, they are translated as their mature size with intrinsic targeting information. Toc75 has been shown to use components of the general import pathway, which include the translocons at the outer and inner envelope membranes of chloroplasts (TOC/TIC) and the stromal processing peptidase (SPP). Other chloroplast β-barrels may use some of these complexes, but this has not been demonstrated. Toc75 is unique in that it requires an additional sorting peptide after its transit peptide (black) that contains a polyglycine stretch and prevents full translocation through the TIC. This peptide is removed by Plsp1, a signal peptidase descended from cyanobacterial LepB. After entering the chloroplast, the β-barrels are likely sorted to OEP80 which is hypothesized to retain the ancestral Omp85 function of β-barrel insertion. Plant genomes contain a *TamB* homolog which may also participate in this process. **c** In *E. coli* multiple sorting pathways for β-barrels have been identified. In both pathways, the nascent β-barrels are translated in the cytosol with signal peptides that direct them to the Sec for post-translational export. The protein is passed to the periplasmic chaperones SurA and/or Skp. The β-barrel assembly module (Bam) is thought to be the pathway used by most β-barrels. This is composed of a central Omp85 homolog (BamA) and several accessory lipoproteins (BamB–E). In contrast, autotransporters are shuttled to the translocation and assembly module (Tam). This is composed of an Omp85 homolog (TamA) and an inner membrane protein (TamB)

a passenger domain that protrudes from or is released by the cell. This complex is composed of TamA, the Omp85 homolog, and TamB, an inner membrane protein that spans the periplasm and contains a domain of unknown function 490 (DUF490) (Selkrig et al. 2012). Although separate Tam and Bam complexes only exist in proteobacteria, TamB homologs are found throughout Gram-negative bacteria, including cyanobacteria (Heinz et al. 2015). Further, the TamB homolog in spirochetes was shown to interact with BamA and is required for proper OM biogenesis (Iqbal et al. 2016). Interestingly, green plants contain a gene that codes for a TamB homolog, At2g25660 in *Arabidopsis thaliana*. This gene is essential for embryo development and its protein product is predicted to contain a chloroplast transit peptide (Hsu et al. 2010; Tzafrir et al. 2004). A point mutation in the rice ortholog was shown to impact the morphology of amyloplasts in several tissues and chloroplasts in some leaves (Matsushima et al. 2014). Although it appears to be important for proper plastid development, a role for plant TamB homolog in β-barrel assembly has not been demonstrated.

The model of β-barrel targeting in cyanobacteria, including the ancestor of chloroplasts, is similar to what is known from other bacteria. The nascent OMPs are translated in the bacterial cytosol with an N-terminal signal sequence, and are exported to the periplasm presumably through the SEC translocase. In the periplasm, the OMP is bound by a cyanobacterial homolog of Tic22 and taken to an Omp85 homolog to be inserted into the OM (Tripp et al. 2012) (Fig. 3a). The cyanobacterial TamB homolog may also play a role, as it does in spirochetes (Iqbal et al. 2016). Other proteins could be involved in this sorting, but their identities are not known.

As the cyanobacteria endosymbiont evolved into an organelle, most of the genes coding for its proteins, including all the OM β-barrels, moved to the nucleus. Like proteins destined for the interior compartments, OM β-barrels must be post-translationally targeted to chloroplast. Since the OM is at the interface of the chloroplast and the cytosol, it is not obvious if the targeting mechanism of β-barrels is similar to other chloroplast proteins. Toc75 was the first chloroplast OM β-barrel whose targeting was studied. Like proteins targeted to the interior compartments, it uses an N-terminal transit peptide and the TOC and TIC (Tranel et al. 1995). Directly following the transit peptide, the precursor of Toc75 contains an envelope retention signal which prevents full translocation through the TIC (Tranel and Keegstra 1996). This envelope retention signal contains a polyglycine stretch that is required for its function (Endow et al. 2016; Inoue and Keegstra 2003). The amount of nascent Toc75 that passes through the OM before membrane insertion is not known. Subsequently, the study of other chloroplast β-barrels including OEP24, OEP21, and OEP40 showed that they were targeted to the chloroplast OM without any processing (Bolter et al. 1999; Harsman et al. 2016; Hofmann and Theg 2005a; Pohlmeyer et al. 1998). This suggests that any targeting information must be intrinsic to the mature protein sequence. OEP80, the paralog of Toc75, was originally thought to reach with chloroplast OM without cleavable targeting information (Inoue and Potter 2004). The coding sequence of the *Arabidopsis thaliana* OEP80 predicts a protein of 80 kDa; however, the size of the protein in chloroplasts was shown to be around 70 kDa (Hsu et al. 2012). This leaves roughly 90 amino acids, which correspond in size to a predicted transit peptide sequence at the N-terminus of OEP80 (Eckart et al. 2002). The region between the first and second methionines (52 amino acids) was shown to be dispensable for targeting and function, but this leaves roughly 40 amino acids as a potential transit peptide (Hsu et al. 2012; Patel et al. 2008). More recently, our work showed that OEP80 was processed to its mature size during in vitro import assays and required ATP for import (Day et al. 2014). These results suggest that OEP80 likely uses a transit peptide for targeting to the chloroplast. Unlike Toc75, the N terminus of OEP80 does not contain a polyglycine stretch, indicating that its envelope sorting mechanism is distinct from Toc75.

Although different chloroplast sorting mechanisms exist for OM β-barrels, it is hypothesized that their membrane insertion converges on the Omp85 homolog, OEP80 (Fig. 3b). If the membrane orientation of OEP80 is conserved with Omp85s in bacteria and mitochondria, chloroplast β-barrels must cross the OM before insertion. Interestingly, Sommer et al. (2011) used transient expression and a split GFP system to show that the POTRA domains and C termini of both Toc75 and OEP80 face the outside of the chloroplast. This change in orientation would allow β-barrels to be directly inserted into the OM from the cytosol. However, this finding was called into question by subsequent studies. Hsu et al. (2012) showed that a T7 tag at the C terminus of OEP80 was protected by the OM in intact chloroplast, suggesting IMS localization. Paila et al. (2016) showed that the POTRA domains of Toc75 interact with Tic22, an IMS localized protein. Lastly, Chen et al. (2016) used an experimental system similar to Sommer et al. (2011) but with stable transformation rather than transient expression to investigate the topology of Toc75. Their results indicated that Toc75's orientation was conserved with other Omp85 homologs. Although they did not investigate the topology of OEP80, they identified problems with the experimental system used by Sommer et al. (2011). Thus, the orientation of OEP80 likely requires its substrates to enter the IMS. Table 1 summarizes our knowledge of known chloroplast OM β-barrels. Except for Toc75 and OEP80, little is known about their targeting. The import of Toc75 requires the general import pathway, so it is possible that β-barrels that do not use transit peptides also enter through the TOC complex (Tranel et al. 1995).

Unlike most bacterial OMs, the chloroplast OM contains α-helical transmembrane proteins. Like most OM β-barrels (excluding Toc75 and OEP80), OM α-helical proteins are translated in the cytosol at their mature size. Alpha helical transmembrane proteins identified so far in the chloroplast OM are predominantly single-pass signal-anchored or tail-anchored (Lee et al. 2014). An ABC-transporter with six transmembrane domains, WBC7, may exist in the OM, however its targeting remains unexplored (Breuers et al. 2011; Schleiff et al. 2003).

Targeting information of chloroplast OM signal-anchored proteins resides in the N-terminal transmembrane domain (Hofmann and Theg 2005b; Lee et al. 2001; Li and Chen 1996; Wu and Ko 1993). Import competition assays suggest that these proteins use some components of the general import pathway (Hofmann and Theg 2005b; Tu et al. 2004). Further, components of the TOC complex were crosslinked to OEP14 during in vitro import (Tu et al. 2004). The role of ATP in the insertion of OM signal-anchored proteins is not clear, with one study suggesting they do not require ATP (Li et al. 1991) and another suggesting they do (Hofmann and Theg 2005b).

Relatively less is known about the insertion of tail-anchored OM proteins, with Toc34, a component of the import apparatus, being the most studied. This protein has a transmembrane domain near the C-terminus that is sufficient for targeting to the chloroplast OM (Li and Chen 1997). Like signal-anchor proteins, conflicting evidence for the involvement of ATP in the insertion of tail-anchored has been obtained (Kessler et al. 1994; Seedorf et al. 1995; Tsai et al. 1999).

The chloroplast targeting of both signal-anchored and tail-anchored proteins is mediated by the cytosolic factor AKR2A (Bae et al. 2008; Dhanoa et al. 2010). AKR2A takes substrates to the chloroplast by specifically recognizing OM lipids (Kim et al. 2014). If and how AKR2A interacts with the protein import apparatus is not known. Table 1 shows several identified OM α-helical proteins. Not all of these have been directly shown to interact with AKR2A, but it is likely that most do.

Conclusion

The chloroplast envelope shares structural and biochemical properties with the ancestral cyanobacterial envelope, but its biogenesis is distinct due to a shift in the direction that its component proteins initially approach their final location. Plants have evolved elegant solutions that combine prokaryotic and novel protein sorting mechanisms to allow this change in direction. This is most evident in the recently identified involvement of an envelope-localized Sec in the insertion of Tic40 and FtsH12 (Li et al. 2017). In bacteria, the Sec facilitates both membrane insertion and translocation. Future work will reveal if this envelope Sec is capable of protein translocation from the stroma to IMS. If so, it is possible that IMS proteins also use a conservative sorting mechanism. OM β-barrels may similarly use a semi-conservative sorting mechanism. In this model, they would enter the IMS through the TOC and then be passed to prokaryotic β-barrel assembly machinery. Recent findings suggest that β-barrel assembly in most bacterial phyla is carried out by a complex containing BamA/TamA and TamB homologs (Heinz et al. 2015; Iqbal et al. 2016). While homologs of both of these proteins exist in chloroplasts, their involvement in chloroplast OM β-barrel assembly has not yet received experimental verification. The model shown in Fig. 3b predicts that OM β-barrels to require two Omp85 homologs for their sorting; Toc75 to enter the IMS, and OEP80 for membrane insertion. Toc75 and OEP80 are paralogs that diverged after endosymbiosis (Day et al. 2014; Topel et al. 2012). Presumably Toc75 evolved from the cyanobacterial Omp85 responsible for β-barrel assembly. Therefore, deciphering the sorting mechanism of chloroplast OM β-barrel proteins could help us understand how Toc75 evolved a new function in general protein import.

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