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Review

The Function and Diversity of Plastid Protein Import Pathways: A Multilane GTPase Highway into Plastids

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The photosynthetic chloroplast is the hallmark organelle of green plants. During the endosymbiotic evolution of chloroplasts, the vast majority of genes from the original cyanobacterial endosymbiont were transferred to the host cell nucleus. Chloroplast biogenesis therefore requires the import of nucleus-encoded proteins from their site of synthesis in the cytosol. The majority of proteins are imported by the activity of Toc and Tic complexes located within the chloroplast envelope. In addition to chloroplasts, plants have evolved additional, non-photosynthetic plastid types that are essential components of all cells. Recent studies indicate that the biogenesis of various plastid types relies on distinct but homologous Toc–Tic import pathways that have specialized in the import of specific classes of substrates. These different import pathways appear to be necessary to balance the essential physiological role of plastids in cellular metabolism with the demands of cellular differentiation and plant development.

Key words: chloroplast, GTPase, plastid, protein import, translocon

The plastids represent a remarkably diverse set of organelles with distinct morphologies and physiological functions within plants (1). Although the role of chloroplasts in photosynthesis is well known, plastids also play essential roles in amino acid, carbohydrate, lipid, and secondary metabolism in all plant tissues. The biogenesis of these organelles is closely linked to the developmental fate of their host cells and relies directly on the import of nucleus-encoded proteins from the cytoplasm following the completion of translation. Genomic and proteomic analyses indicate that 2500–4000 different plastid proteins are imported into these organelles (2–4). In *Arabidopsis thaliana*, this represents 10–15% of the entire nuclear genome. Recent developments from studies of protein import into plastids are revealing the mechanistic details of preprotein recognition, membrane translocation, and maturation during the import process. Although these studies have been largely restricted to chloroplasts, they have led to the discovery of a set of homologous, yet distinct, pathways that provide a means of balancing the capacity and selectivity of protein import with the demands imposed by rapidly changing gene expression profiles that accompany the events of plant development. Several excellent recent reviews have been published that focus on the components of the import process and their proposed functions in trafficking proteins into the organelle (5–8). In this review, we will incorporate a discussion of the multiplicity and evolutionary origins of import pathways, as this relatively new information provides insight into the molecular basis of pathway specificity and the mechanism of membrane translocation. We will limit our discussion to the events at the plastid envelope that result in the transport of proteins from the cytoplasm into the organelle. Trafficking to the chloroplast thylakoid membrane will not be discussed in detail, because it occurs subsequent to and independent of import into the organelle (8), and space limitations preclude a fair treatment of both protein import and thylakoid targeting.

All plastids are bound by a double membrane envelope that corresponds to the double membrane of the gram-negative cyanobacterial relative that served as the original endosymbiont at the advent of plant evolution. Two membrane protein complexes at the envelope associate to provide a direct conduit for nucleus-encoded proteins from the cytoplasm to the interior stromal compartment. The translocon at the outer envelope membrane of chloroplasts (Toc complex) mediates the initial recognition of preproteins and their translocation across the outer membrane (9). Outer membrane translocation requires ATP and GTP in the intermembrane space and exterior, respectively (10–12). The translocon at the inner envelope membrane of chloroplasts (Tic complex) physically associates with Toc complexes and provides the membrane translocation channel for the inner membrane. In addition, the Toc and Tic complexes interact with a set of molecular chaperones in the exterior and interior of the organelle that appear to maintain preproteins in an unfolded import-competent state, participate as part of the driving force for protein translocation, and assist in the folding and assembly of newly imported proteins (13–15). As such, the plastid

protein translocons possess the three basic components found in all membrane translocation systems: preprotein receptors linked to protein-conducting channels and an associated energy-dependent membrane translocation motor (16). Despite the adherence to the general principles of protein trafficking, the molecular details of import have revealed a novel trafficking system that possesses remarkable flexibility in adapting to the physiological and developmental demands on plastid function.

Targeting to the Chloroplast Surface

The vast majority of nucleus-encoded plastid preproteins are synthesized with a cleavable amino-terminal transit peptide that is necessary and sufficient for targeting to the organelle (17,18). The diversity in transit peptide size (20 to >70 amino acids) and the lack of sequence conservation have perplexed the field for decades, thwarting efforts to define the intrinsic structural determinants that account for targeting specificity. The similarities among transit peptides are limited to a deficiency in acidic and enrichment in hydroxylated amino acids, giving them an overall basic charge. In contrast to the α-helical presequences of mitochondrial preproteins, the biophysical analysis of a limited number of transit peptides has not revealed any regular secondary structural elements in aqueous solutions. The recent discovery of multiple, distinct Toc complexes that differ in their transit peptide receptor components (19,20) could account for the inability to define consensus elements across all transit peptides. The Toc receptors appear to recognize distinct classes of preproteins (see below), and it appears likely that an analysis of transit peptides among members of each specific class will be more fruitful in identifying common recognition features.

The preprotein receptors at the chloroplast surface were first defined as protease-sensitive outer membrane proteins essential for preprotein binding and import (21–23). Using chemical cross-linking, antibody inhibition, or arrested translocation intermediates (21–24), three distinct proteins were identified that are now considered the core components of the Toc complex and are sufficient to reconstitute protein translocation in artificial lipid vesicles (25) (Figure 1). The core Toc complex consists of two homologous GTP-binding proteins, Toc159 and Toc34, and a channel protein, Toc75, related to prokaryotic solute channels. Both Toc159 and Toc34 are exposed at the chloroplast surface and were shown to interact with preproteins at the earliest stage of import (24,26). In the absence of added ATP and GTP, Toc34 and Toc159 can be chemically cross-linked to bound preproteins with Toc159 specifically interacting with the transit peptide. In the presence of external ATP and GTP, preproteins cross the outer membrane (27). At this stage, the mature regions of preproteins can be chemically cross-linked both to Toc159 and Toc75 (24,26). These data suggest that Toc159 functions both as a primary preprotein receptor and in conjunction with the protein-conducting channel to promote membrane translocation. Toc34 also has been shown to directly bind to preproteins (28,29), supporting its role in preprotein recognition at the chloroplast surface.

The hallmark structural feature of the Toc159 and Toc34 receptors is a conserved GTP-binding domain (G-domain). Toc34 consists almost entirely of the G-domain, anchored at the surface of the outer membrane by a stretch of hydrophobic amino acids at its C-terminus. Toc159 has a central G-domain flanked by an N-terminal acidic domain (A-domain) and C-terminal membrane-anchoring domain (M-domain). The crystal structure of the soluble G-domain of Toc34 revealed a GDP-bound dimer (30), suggesting that nucleotide-dependent GTPase dimerization could play a central role in the molecular mechanism of translocon function (31). *In vitro* binding studies also demonstrate a direct interaction between Toc34 and Toc159 that involves dimerization of their G-domains (32,33). Moreover, the binding studies reveal a preferential interaction between the two G-domains in the presence of GDP suggesting that the interaction is regulated through GTP binding and hydrolysis. These data have led to two models in which nucleotide-dependent Toc GTPase dimerization regulates a molecular gate that controls preprotein access to the translocation channel (34–36) (Figure 2). Preprotein binding has been shown to stimulate the GTPase activities of the Toc receptors (29,37,38), consistent with the proposal that preprotein recognition opens the gate to the translocon and triggers its transfer to the protein-conducting channel. In one such model, the targeting hypothesis (34,35) (Figure 2A), the primary role of GTP is to regulate Toc GTPase dimerization and thereby act as a molecular switch that controls the fidelity of preprotein targeting to the translocon channel. The targeting hypothesis proposes that Toc159 is the primary preprotein receptor, conferring specificity on the import pathway, whereas Toc34 acts in concert to promote transfer of preproteins to the translocation channel. In the second model, the motor hypothesis (25,36) (Figure 2B), GTP hydrolysis at Toc159, not only acts as part of a gating mechanism; it also is envisioned to drive a repetitive motor activity within the receptor that pushes preproteins across the membrane. Both models agree that the GTPase activities of the Toc receptors play a key role in regulating the commitment of preproteins to import, and they both hypothesize that GTP acts by regulating receptor dimerization. Although a role for GTP is clear, direct evidence for the participation of homodimerization or heterodimerization in translocon gating is lacking. Several pieces of evidence are inconsistent with the role of Toc159 as a GTP-driven motor. *In vivo* and *in vitro* evidence demonstrates that the Toc159 M-domain can at least partially substitute for the activity of the native protein (39,40). Furthermore, GTP hydrolysis is not required once the N-terminal

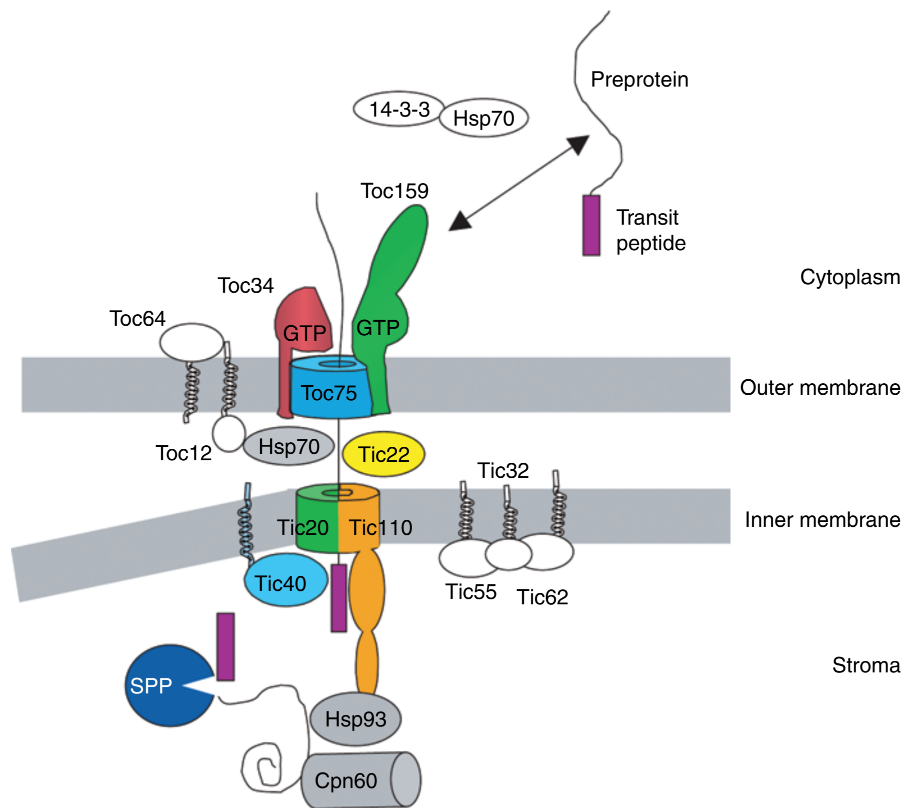

Figure 1: Components of the chloroplast protein import machinery. Components of the translocon at the outer and inner membrane are designated as Toc and Tic, respectively, followed by their molecular masses in kDa. The core components of the import machinery are shown in color. Components that are proposed to perform accessory or regulatory functions are shown in white. Nucleus-encoded preproteins first insert across the outer membrane via the activities of the core Toc complex, consisting of the Toc GTPase receptors, Toc159 and Toc34, and the channel component, Toc75. In some cases, preprotein targeting is proposed to involve a cytosolic guidance complex, composed of a 14-3-3 protein and an Hsp70 chaperone. Upon insertion across the Toc complex, preproteins bind to an Hsp70 chaperone in the intermembrane space and are directed to the Tic complex with the assistance of Tic22. At this point, the Toc and Tic complexes associate, and translocation proceeds simultaneously across both membranes. Tic110 and Tic20 are proposed to form the Tic channel. The stromal domain of Tic110 functions as a molecular scaffold by binding the preprotein and recruiting chaperones (Hsp93 and Cpn60) with the assistance of Tic40 for subsequent folding of the fully imported preprotein. The stromal processing peptidase (SPP) cleaves the transit peptide from the preprotein during or shortly after import. The Tic55, -32, and -62 components are proposed to regulate the import of specific substrates via a redox cycle.

region of the preprotein is transferred into the translocon channel (11).

Additional and complementary evidence for Toc GTPase function has been obtained using *in vivo* approaches in *Arabidopsis*. The *Arabidopsis* homologs of pea Toc34 and Toc159 are encoded by small gene families, containing two (atToc33 and atToc34) and four genes (atToc159, -132, -120, and -90) (41). AtToc33 and atToc159 are considered the orthologs of pea Toc34 and Toc159, respectively. AtToc33 and atToc159 are considered the orthologs of pea Toc34 and Toc159, respectively. Null mutants of both atToc33 (*ppi1*) (42) and atToc159 (*ppi2*) (43) demonstrate that both genes are involved in chloroplast biogenesis. Furthermore, the analysis of mutants lacking GTP-binding activity indicates an essential role for GTP at both atToc33 (28) and atToc159 (37,40). GTP-binding mutants of atToc159 are abnormally localized to the cytosol (32,40,44), suggesting a dominant role of the G-domain in targeting atToc159 to chloroplasts *in vivo* and *in vitro*. The detection of a soluble form of atToc159 has led to the proposal that it might function as a cycling receptor that delivers bound preproteins from the cytoplasm to the chloroplast surface (32,40,44). However, direct evidence for such a function is lacking. The involvement of Toc34 and Toc159 in preprotein binding and the role of GTP in membrane insertion demonstrate a key role for the co-ordinate activities of the two GTPases in preprotein recognition and the initiation of membrane translocation at the translocon. However, it is clear that activities of the GTPases are more complex than the current models suggest. A more detailed analysis of GTPase mutants *in vivo*, specifically those that possess specific defects in nucleotide binding or hydrolysis, should provide more detailed evidence for the roles of GTP in regulating Toc-complex assembly and preprotein recognition.

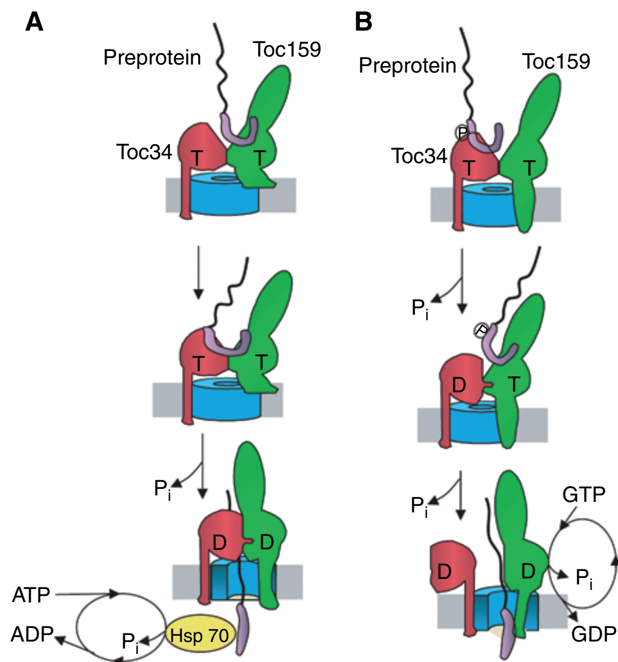

Figure 2: Two models for preprotein targeting to the Toc complex. A) The 'targeting' hypothesis proposes that Toc159-GTP (T) serves as the primary preprotein receptor and that Toc159-GTP (T) and Toc34-GTP (T) interact co-operatively via their GTPase domains to form a GTP-regulated gate to the translocon. Preprotein binding activates GTP hydrolysis, converting the receptors into the GDP-bound state (D). Hydrolysis is proposed to induce a conformational change in the GTPase interaction that promotes insertion of the preprotein into the translocation channel. Preprotein translocation is driven by an ATP-dependent cycle at the intermembrane space Hsp70. B) The 'motor' hypothesis proposes that Toc34 in its GTP-bound state (T) acts as the initial receptor by binding to the phosphorylated form of the preprotein transit peptide. Toc34 is converted to its GDP-bound state (D) by preprotein-stimulated GTP hydrolysis, resulting in transfer of the preprotein to Toc159-GTP (T). Following dephosphorylation, the preprotein is driven across the outer membrane through the Toc75 channel via a GTP-dependent Toc159 motor. In both models, the Toc159 and -33 receptors are reset to their GTP-bound state and are ready for further translocation cycles.

Chemical cross-linking (24,26) and proteoliposome reconstitution studies (45) provide compelling evidence for Toc75 as a major component of the protein-conducting channel of the Toc complex. Toc75 is essential (46) and is predicted to form a β-barrel-type membrane protein with a structure distantly related to transporters found in the outer membranes of gram-negative bacteria and the Tom40 channel of the mitochondria outer membrane preprotein translocase (47). Electrophysiological studies of reconstituted Toc75 reveal a channel of approximately 14 angstroms (45), one large enough to accommodate an unfolded polypeptide chain. Conductivity through the channel is responsive to the presence of preproteins, consistent with studies indicating that Toc75 possesses a preprotein-binding site. Toc75 cannot mediate membrane translocation of preproteins on its own (25) but relies on the co-ordinate activities of the Toc GTPases and an hsp70-type chaperone in the intermembrane space to catalyze transport. Whereas the Toc GTPases serve roles as preprotein receptors and regulators of the initiation of translocation, the hsp70 is proposed to function as a driving force for outer membrane translocation, consistent with the ATP requirement for translocation. The hsp70 also is envisioned to maintain the requisite unfolded conformation of the preprotein during translocation and prevent mistargeting to the intermembrane space before the Tic translocon is engaged in the import process. An outer membrane protein, Toc12, containing a DnaJ domain has recently been identified and has been proposed to co-ordinate the activity of the envelope hsp70 at import sites (48).

Several studies suggest that targeting to the Toc translocon might be regulated. Both Toc33 (29) and the transit peptide of at least one preprotein (49,50), the small subunit of rubisco (preSSU), can be phosphorylated *in vitro*. A 'guidance complex' in wheat germ extracts that contains a 14-3-3 protein and a cytosolic hsp70 recognizes the phosphorylated form of preSSU (50). This complex is proposed to facilitate targeting to the Toc translocon by binding to an outer envelope membrane protein, Toc64, which is loosely associated with the trimeric Toc complex. A phosphorylation–dephosphorylation cycle involving the preprotein and Toc33 has been proposed to regulate the transfer of preproteins into the Toc channel (36). To date the *in vivo* role for phosphorylation of the Toc GTPase or preproteins has not been demonstrated. The phosphorylation site of preSSU is absent in many species, and mutation of the site in *Arabidopsis* has no apparent effect on import (51). Furthermore, Toc64-null mutants exhibit no apparent phenotypes in *Physcomitrella* (52). Therefore, phosphorylation might play a specialized regulatory role rather than an integral part of the general targeting pathway.

Inner Membrane Translocation

A combination of genetic and biochemical approaches has identified a number of candidates for components of the Tic translocon (Figure 1). However, a stable Tic complex containing stoichiometric amounts of the Tic components has not been isolated. Furthermore, several known components appear to associate in response to preprotein import, suggesting that the formation of the inner membrane translocon is dynamic. Immuno-electron microscopic localization of import intermediates and biochemical analyses of import complexes demonstrate that Toc and Tic components physically associate early in the import process (14,53). This association occurs at membrane-contact sites between the outer and inner envelope and appears to involve the direct binding between Toc and Tic components. A peripheral inner membrane protein, Tic22, has been proposed to facilitate this interaction,

although the precise molecular interactions and their regulation have not been studied in detail (54). Several of the Tic components are found associated with one another in these Toc–Tic supercomplexes, leading to the hypothesis that the inner membrane translocon is formed by assembly with Toc components in response to preprotein translocation across the outer membrane.

The dynamic nature of Tic complexes has focused the analysis of inner membrane translocation on the activities of individual components. These studies have revealed a central role for Tic110 in assembly of active translocons and in preprotein translocation across the inner membrane. Tic110 is an abundant inner membrane protein, and approximately 5% of the protein is found stably associated with Toc components in Toc–Tic supercomplexes (14,54). It is encoded by a single gene in all species examined and is essential for *Arabidopsis* viability (55,56), underscoring its critical role in plastid biogenesis and protein import. Tic110 contains two short α-helical membrane anchors at its N-terminus and a large (approximately 95 kDa) stromal domain (57,58). It covalently cross-links to preproteins during import, and its stromal domain contains a transit peptide-binding site (58). These data implicate Tic110 directly in the translocation reaction. In addition, deletion analysis of the Tic110 stromal domain *in vivo* and *in vitro* demonstrates that it contains determinants that are required for the assembly of a functional inner membrane translocon within supercomplexes (55). Tic110 also associates with Tic40 (59), an inner membrane protein containing a DnaJ domain, a plastid hsp93 chaperone (14), and the plastid GroEL homologue (13). These activities have led to the proposal that Tic110 provides a docking site for preproteins, as they insert across the inner membrane and forms a scaffold for the assembly of the molecular chaperone machinery that drives preprotein translocation and assists in subsequent folding or suborganellar targeting of newly imported proteins.

At least two candidates have been identified as possible channel components for the Tic translocon. One of these is Tic110. Its abundance relative to other known Tic components and its interaction with preproteins are consistent with this activity. The incorporation of a denatured fragment containing a portion of the stromal domain of Tic110 into proteoliposomes gave rise to a membrane channel (60). However, a similar structure is not found in native Tic110 or in similar Tic110 fragments when expressed *in vivo* (58). Therefore, it appears unlikely that the stromal domain forms a component of the membrane channel directly, although other regions (i.e. the membrane anchors) might participate in channel formation. A second component, Tic20, has been implicated in channel formation. Tic20 is distantly related to the Tim17/23 channels of the mitochondrial inner membrane import machinery, and the reduction of Tic20 levels with antisense RNA in *Arabidopsis* specifically disrupts preprotein translocation across the inner membrane and leads to defects in chloroplast biogenesis (54,61). Tic20 is detected in Toc–Tic supercomplexes in association with Tic110 (54). Therefore, it is possible that the interaction of these two components results in the formation of the Tic channel.

The role of molecular chaperones in providing the driving force for translocation across the inner membrane is consistent with the requirement for ATP hydrolysis in the stroma. In contrast to mitochondria and the ER, the primary chaperone activity does not appear to involve a member of the hsp70 family. Instead, the plastid hsp93 is stoichiometrically associated with Tic110 (14,15). Mutants in the major form of the two plastid hsp93 genes are defective in protein import (62,63), consistent with a direct role in Tic function. Although there is no direct evidence for the role of hsp70-type chaperones at the inner membrane, the presence of the J-domain-containing inner membrane protein, Tic40 (59), and the association of preproteins with the stromal hsp70 shortly after import (64) suggest a role for this chaperone family downstream of the import process. Null mutants of Tic40 in *Arabidopsis* are viable and exhibit moderate defects in chloroplast biogenesis (59). Furthermore, Tic40 has not been shown to bind molecular chaperones directly. Nonetheless, the Tic40 mutants exhibit reduced protein import efficiency, raising the possibility that an hsp70 chaperone activity might assist in the import of a subset of preproteins.

During or shortly after import, the transit peptide of preproteins is cleaved by a soluble stromal processing peptidase (SPP) (65). SPP is not tightly associated with the inner membrane or Tic components and therefore does not appear to participate directly in translocon function. SPP is a member of the zinc metallopeptidase family M16 (66), and its activity is essential in *Arabidopsis* (67). It processes transit peptides in a multistep reaction that both cleaves the peptide from the precursor and degrades it to subfragments that are subsequently hydrolyzed by an ATP-dependent protease (68). Following processing, newly imported proteins fold and assemble, often with the assistance of Cpn60, the plastid GroES-GroEL chaperonin complex, or undergo additional suborganellar targeting to their appropriate final residence.

Several recent observations suggest that protein import can be directly regulated. A set of redox proteins (Tic55, Tic62, and Tic32) has been shown to associate with Tic complexes (69,70). Furthermore, the import of at least one substrate, the precursor to non-photosynthetic ferredoxin, is regulated by the photosynthetic state of plastids (71). The redox state of chloroplasts is directly related to photosynthetic activity and is co-ordinated with plant growth activities. Therefore, a redox regulatory mechanism might serve to co-ordinate import activities with the metabolic activities of the organelle.

Targeting to the Envelope Membranes

Plastids are exceptionally complex organelles. Chloroplasts contain at least six suborganellar compartments: outer and inner membranes, intermembrane space, stroma, and thylakoid membrane and lumen, all of which require specific trafficking systems. The targeting systems of the thylakoid system have been studied in great detail, and all appear to be conserved from the protein export systems that exist in gram-negative bacteria (8). The mechanisms of targeting nucleus-encoded proteins to the chloroplast envelope have only recently been investigated in detail. Most outer envelope membrane proteins lack transit peptides and are targeted by signals contained within their membrane anchors (e.g. Toc64, Toc33/34, and OEP14) (72–75). Recent studies on a small outer membrane protein, OEP14, suggest that its integration involves the Toc75 channel but does not require the assistance of the Toc GTPases or ATP (76). These data suggest that Toc75 might form a common translocation channel for proteins that cross or integrate into the outer membrane. The participation of Toc75 in multiple targeting processes could explain why it is found in significant molar excess relative to the Toc GTPases, with a large fraction (approximately 50%) not associated with the other known Toc components (54).

Toc75 itself is unique in that it possesses a bipartite transit peptide, the N-terminal half of which functions similar to a standard transit peptide (77,78). The C-terminal region contains a polyglycine stretch and functions as a stop-transfer signal that triggers integration of Toc75 into the outer membrane (79). Therefore, it appears that Toc75 initially engages the trimeric Toc complex en route to the outer membrane. The mechanism of integration of Toc75 into the membrane is not understood. To date, components similar to the SAM complexes that mediate the integration of β-barrel proteins into the mitochondrial outer membrane (79) have not been identified in plastids.

Our knowledge of targeting to the envelope intramembrane space has been obtained largely from studies on Tic22 (80). It contains a cleaved N-terminal-targeting signal but does not compete with the import of stromal proteins. Therefore, it does not appear to use the identical pathway as proteins possessing transit peptides. Targeting to the inner membrane has been examined most extensively in the case of Tic110. PreTic110 contains a transit peptide and utilizes the Toc–Tic system for import (81,82). Targeting to the inner membrane appears to require signals within the transmembrane regions of the protein. However, studies using preTic110 chimerical proteins suggest that Tic110 might use a stromal intermediate en route to the inner membrane (82). Furthermore, dominant negative mutants of Tic110 that disrupt Tic complex formation result in the accumulation of normal Tic110 in the stroma (55). These data suggest that at least a subset of inner membrane proteins is reinserted into the membrane from the stroma after import. It is not known whether a co-translocational targeting pathway for inner membrane proteins, similar to the Tim22 pathway in mitochondria (83), also exists in plastids.

Adaptation of Translocon Function to Developmental or Physiological Conditions

The discovery of multiple, developmentally regulated Toc translocons that mediate the import of distinct classes of preproteins is one of the most intriguing observations in recent studies on plastid import (Figure 3). The *ppi2* and *ppi1* mutants have not only provided new mechanistic insights but have also provided the first inroads into the physiological role of the members of the Toc-GTPase family. *Ppi1* and *ppi2* exhibit pale green and albino phenotypes, respectively, indicative of a specific defect in chloroplast biogenesis (42,43,84,85) (Figure 3). *Ppi2* plants lack developed chloroplasts at the ultrastructural level. Moreover, *ppi2* plants fail to accumulate many of the normally abundant photosynthetic proteins owing to the repression of nuclear gene expression caused by the defective organelle. In contrast to photosynthetic proteins, many other plastid proteins accumulate normally in *ppi2* plastids. These proteins have been operationally defined as 'housekeeping' proteins. AtToc159 apparently is not required for the import of the housekeeping proteins. Instead, it has been proposed that the remaining homologs of atToc159, atToc132, -120, and -90 mediate the import of constitutively expressed housekeeping proteins (43) (Figure 3). Analysis of the corresponding null mutants has by and large substantiated the hypothesis (19,86,87). Double knockout mutants of atToc132 and atToc120 were found to be either embryo or seedling lethal, which is consistent with their proposed role in the import of essential housekeeping proteins (19,87). Indeed, the levels of housekeeping proteins were reduced in mutant plants lacking only atToc132 alone. Evidence for an involvement of the Toc159 homologs in independent import pathways was obtained through the complementation experiments; ectopic expression of atToc159 was unable to complement the atToc132/120 double knockout and vice versa (19,87). In contrast, the visible phenotypes of the atToc33- (*ppi1*) and atToc34-null mutants are relatively mild or nonexistent, respectively (20,88). Moreover, the expression of Toc34 under a strong promoter complemented the atToc33 *ppi1* mutant, indicating a functional overlap between the two proteins. The double knock out of atToc33 and atToc34, however, resulted in embryo lethality pointing to a single essential function of the two proteins (88). In summary, the data suggest that the Toc159 homologs are the key determinants of import substrate specificity, although the discerning features of the transit sequences of photosynthetic and housekeeping proteins have not been defined. AtToc33 and atToc34

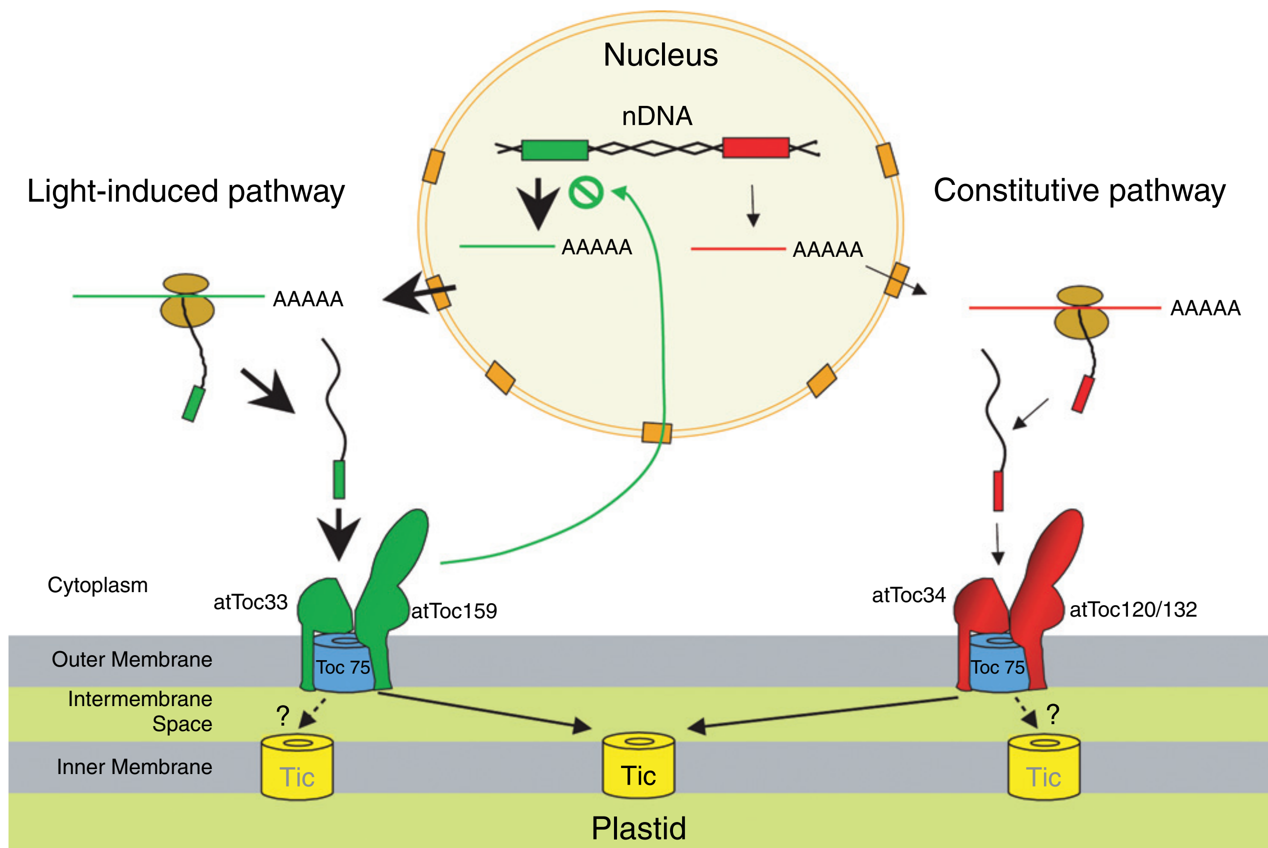

Figure 3: Alternative import pathways in *Arabidopsis thaliana*. At least two distinct preprotein import pathways exist that are distinguished by the presence of unique Toc GTPase receptors. The 'constitutive pathway' is proposed to mediate the import of housekeeping proteins that are essential for plastid function in all tissues. This pathway is defined by a distinct Toc complex containing the unique atToc34 and atToc120/132 GTPase receptors. AtToc120/132-null mutants are embryo or seedling lethal consistent with a role for this pathway in the essential housekeeping functions of plastids. The 'light-induced pathway' is developmentally regulated and is required for the import of preproteins that are essential for the biogenesis of chloroplasts and the photosynthetic apparatus. This pathway is defined by Toc complexes containing the atToc33 and atToc159 receptors. Many photosynthesis-associated proteins are deficient in mutants lacking atToc159 (*ppi2*) or atToc33 (*ppi1*), whereas non-photosynthetic proteins accumulate normally. The light-induced pathway is closely linked to the transcriptional regulation of genes encoding photosynthetic proteins. Disruption of this pathway results in transcriptional repression of nuclear genes required for chloroplast biogenesis.

appear to be less discriminating with regard to the two operationally defined classes of import substrates. A role of the Toc159 homologs as the determinants of substrate specificity is consistent with their proposed function as primary preprotein receptors. Likewise, the overlapping substrate specificity of atToc33 and atToc34 suggests a function as co-receptors with the Toc159 family members. The Toc75 channel protein is encoded by single, essential gene in *Arabidopsis* (46). It is therefore conceivable that atToc33 and atToc34 may function as molecular match-makers between atToc75 and atToc159 family members to assemble different Toc translocons with distinct substrate specificities in addition to their essential role in substrate recognition.

Analyses of the genomes of several cyanobacterial species and early plastid-bearing species are consistent with the proposal that the Toc GTPases are regulators of the import process that evolved to confer developmental and tissue specificity on the import process (89). No homologous GTPases are found in cyanobacteria, although homologues of Toc75, Tic20, and Tic22 are present. Furthermore, the genomes of simple plastid-bearing species contain Toc33/34 homologues but lack the larger Toc159 family proteins. The Toc159 family of GTPases appears later in evolution in organisms with more complex metabolic and developmental programs (e.g. tissue specialization and photomorphogenesis). These observations are consistent with the hypothesis that the import system was derived from an existing membrane transport system in cyanobacteria and was modified with the acquisition of novel regulators that would confer specificity and directionality on the import process. Consistent with the genetic studies in *Arabidopsis*, the Toc34 GTPases would serve as essential, relatively indiscriminant preprotein receptors that mediate the targeting of a wide variety

of plastid proteins. In simple photosynthetic eukaryotes, this activity would be sufficient for organelle function and maintenance. More complex physiological responses and the evolution of multicellular plants with multiple plastid types required the acquisition of the Toc159 family members and the resultant advent of different import pathways. This provided a mechanism to discriminate between classes of preproteins and balance import with expression profiles that were spatially and temporally regulated.

It remains to be determined whether or not multiple Tic translocons exist, as is the case for targeting for protein trafficking at the inner mitochondrial membrane (83). The fact that Tic110 is an essential protein (55,56) suggests that it is a common component of most if not all import pathways. This idea is supported by the observation that it associates with Toc–Tic supercomplexes containing any of the Toc GTPase components (19). Furthermore, a Tic110 gene is present in the simplest photosynthetic eukaryotes, indicating that its activity was required early in endosymbiosis. It is conceivable that the dynamic assembly of the Tic complexes is required to bring together common (e.g. Tic110) and specialized components (e.g. Tic55 and Tic32) to form translocons with distinct roles in targeting.

Future Directions

The application of elegant biochemical techniques has revealed the core constituents of the Toc–Tic import machinery, and the more recent use of reverse genetic approaches in *Arabidopsis* and the moss, *Physcomitrella* (90), has begun to place these components in a physiological context and test their functions *in vivo*. Several aspects of the *in vitro* and *in vivo* results remain to be reconciled. For example, it now should be possible to test the relative roles of nucleotide binding and hydrolysis to examine the roles of the Toc GTPases in preprotein recognition and the energetics of membrane translocation, thereby testing elements of both the targeting and motor hypotheses. The mechanism of Toc GTPase function remains one of the most intriguing aspects of the import reaction, and like other trafficking GTPases (i.e. Ran, Rabs, and SRP), studies on the plastid proteins are likely to continue to yield unexpected results that will render the simple models obsolete.

An understanding of the structure and dynamics of the Tic complex remains another challenge for future investigations. Does the apparent instability of Tic translocon(s) represent an artifact of biochemical manipulation or reflect the active assembly of translocons in response to import? This dynamic could be essential to maintain the integrity of the inner membrane in the absence of protein translocation. Alternatively, the dynamics could be necessary to combine sets of common components (e.g. Tic110) with specialized proteins (e.g. regulators) to assemble functionally distinct translocons similar to the diverse set of Toc complexes.

Another particularly exciting aspect of plastid protein trafficking derives from the proteomic identification of a number of internal plastid proteins that do not contain cleaved transit peptides (2). The molecular details of the import of these proteins remains to be investigated, but it appears that they utilize one or more distinct routes into the organelle (91,92). Furthermore, the import of at least one protein with a cleavable transit peptide (protochlorophyllide oxidoreductase A) is tightly regulated and appears to involve auxiliary regulatory proteins (93,94). Thus, it is clear that the concept of a single, general import pathway consisting of the known Toc and Tic components is outdated. Defining the specific roles of multiple import pathways will not only be interesting from a mechanistic standpoint, but it also represents an important aspect of placing the variety of trafficking pathways in the context of the complex developmental and physiological functions of diverse plastid types that reside throughout plant tissues.

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