

DARWIN REVIEW

Origins, function, and regulation of the TOC–TIC general protein import machinery of plastids

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Abstract

The evolution of chloroplasts from the original endosymbiont involved the transfer of thousands of genes from the ancestral bacterial genome to the host nucleus, thereby combining the two genetic systems to facilitate coordination of gene expression and achieve integration of host and organelle functions. A key element of successful endosymbiosis was the evolution of a unique protein import system to selectively and efficiently target nuclear-encoded proteins to their site of function within the chloroplast after synthesis in the cytoplasm. The chloroplast TOC–TIC (translocon at the outer chloroplast envelope–translocon at the inner chloroplast envelope) general protein import system is conserved across the plant kingdom, and is a system of hybrid origin, with core membrane transport components adapted from bacterial protein targeting systems, and additional components adapted from host genes to confer the specificity and directionality of import. In vascular plants, the TOC–TIC system has diversified to mediate the import of specific, functionally related classes of plastid proteins. This functional diversification occurred as the plastid family expanded to fulfill cell- and tissue-specific functions in terrestrial plants. In addition, there is growing evidence that direct regulation of TOC–TIC activities plays an essential role in the dynamic remodeling of the organelle proteome that is required to coordinate plastid biogenesis with developmental and physiological events.

Keywords: Chloroplast biogenesis, chloroplast protein import, protein targeting, protein quality control, transit peptide, endosymbiosis.

Introduction

In vascular plants, 2000–3000 nuclear genes are required for plastid function, with only ~120 genes retained in the plastid genome (Jarvis and Lopez-Juez, 2013). The translocon at the outer chloroplast envelope (TOC)–translocon at the inner chloroplast envelope (TIC) system mediates the import of the vast majority of nuclear-encoded plastid proteins from the

cytoplasm, and it is therefore referred to as the general import system (Richardson *et al.*, 2017; Bölter, 2018; Day and Theg, 2018). Although many mechanistic details of the general import system remain to be defined, the known activities of the core TOC–TIC components fulfill all of the criteria for a functional protein import system (Fig. 1). Nuclear-encoded plastid

Abbreviations: BAM, β -barrel assembly machinery; Cdc48, cell cycle division 48; CHIP, C-terminus of Hsp70-interacting protein; ERAD, endoplasmic reticulum-associated degradation; GA, gibberellic acid; GAD, GTPases activated by dimerization; HSF, heat shock factor; Hsp, heat shock protein; KOC, kinase of the outer chloroplast membrane; Mg-ProtoIX, Mg-Protoporphyrin IX; OMP, outer membrane protein; POTRA, polypeptide transport-associated; ppl, plastid protein import mutant; PUB4, U-box domain-containing protein 4; ROS, reactive oxygen species; SP, suppressor of *ppl1*; SRP, signal recognition particle; TAM, translocation and assembly module; TIC, translocon at the inner chloroplast envelope; TOC, translocon at the outer chloroplast envelope; UPS, ubiquitin–proteasome system; Ycf, hypothetical chloroplast ORF.

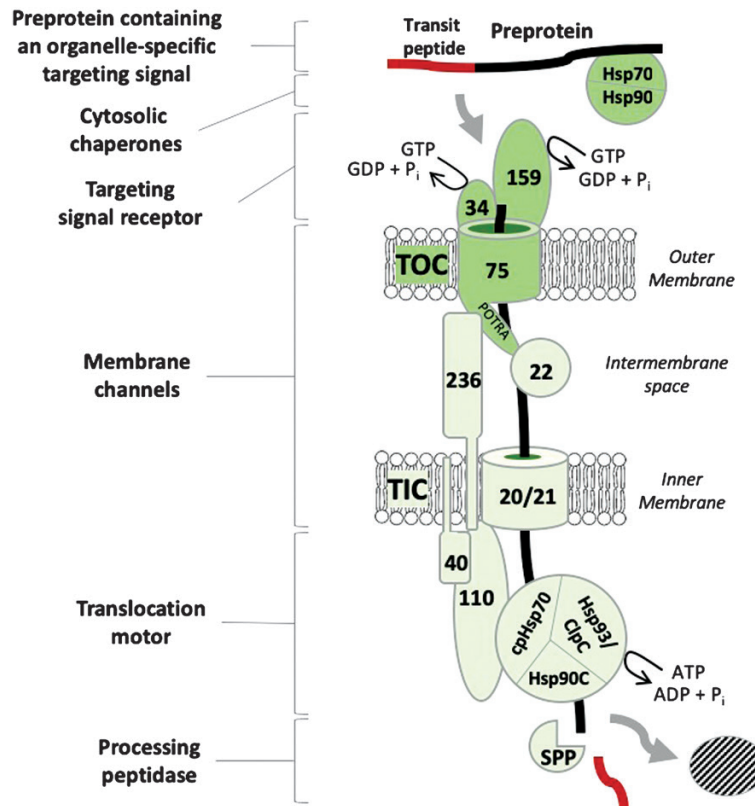


Fig. 1. The core components of the TOC–TIC general import machinery of chloroplasts. The core components of the general import machinery are conserved across the green lineage. The newly synthesized preprotein is targeted to the TOC complex at the outer membrane by binding of its intrinsic transit peptide to the Toc34 (34) and Toc159 (159) receptors. Targeting is aided by cytosolic chaperone complexes of the Hsp70 and Hsp90 families. The GTPase activities of the receptors function as a checkpoint for the commitment of the preprotein to transport through the TOC and TIC membrane channels formed by Toc75 (75) and Tic20/21 (20/21), respectively. TOC–TIC supercomplexes formed by the binding of Tic236 (236) to Toc75 and Tic110 (110) at the TIC complex facilitates direct transport of the preprotein from the cytoplasm to the stroma. Mistargeting to the intermembrane space is avoided by the combined chaperone activities of the Toc75 POTRA domains and Tic22 (22). Tic20/21 form the major components of the TIC import channel, and they associate with Tic110 and Tic40 (40), which form a scaffold for the assembly of the ATP-dependent import motor in the stroma. The import motor drives unidirectional translocation of the preprotein via the combined activities of cpHsp70, Hsp93/ClpC, and Hsp90C chaperones. Upon import, the transit peptide is cleaved by the stromal processing peptidase (SPP). The reader is referred to other recent reviews for more comprehensive descriptions of accessory components associated with the general import machinery.

proteins contain intrinsic targeting signals to direct them to the organelle. Cytosolic factors and protein import receptors at the chloroplast surface specifically recognize the targeting signals and direct preproteins to the organelle. Targeting is assisted by cytosolic chaperones that prevent misfolding or aggregation in the cytoplasm. The targeting receptors are coupled to membrane channels, which mediate transport across the organelle membrane. In turn, the membrane channels associate with an energy-dependent import motor that provides the driving force for transport from the cytoplasm into the stroma. In the case of plastids, membrane channels in the outer and inner membrane work in concert to facilitate transport across the envelope and prevent mistargeting to the intermembrane space.

Protein import systems with functions comparable with TOC–TIC have not been identified in cyanobacteria or other Gram-negative bacteria (Day and Theg, 2018). Although the major bacterial protein targeting systems, including the Sec, Tat, and signal recognition particle (SRP) pathways, were conserved in chloroplasts, they do not participate in protein import, but were modified to participate in the biogenesis of internal membrane systems, such as the thylakoids and inner

envelope membrane (Day and Theg, 2018). It therefore appears that the plastid protein import system was a novel adaptation to enable endosymbiosis. Despite their unique functions, we now know that core components of the TOC–TIC general import system were also derived from specialized bacterial protein targeting systems and repurposed to constitute the protein import apparatus. Here, we provide a brief overview of the mechanism of protein import, but our focus will be on highlighting the origins of the core, conserved components of the TOC–TIC systems that constitute the general import system. We will also highlight recent studies that reveal how diversification and regulation of the general protein import machinery in terrestrial plants contribute to the control and functional remodeling of plastids during developmental and physiological changes. The reader is referred to a number of recent reviews that focus on the mechanism of protein import for a more comprehensive description of additional components associated with the general import apparatus (Paila *et al.*, 2015; Chotewutmontri *et al.*, 2017; Lee *et al.*, 2017; Richardson *et al.*, 2017; Sjuts *et al.*, 2017; Bölter, 2018; Day and Theg, 2018; Schwenkert *et al.*, 2018).

Overview of TOC–TIC function

Nuclear-encoded plastid proteins are translated as preproteins on cytoplasmic ribosomes, containing intrinsic transit peptides that serve as the targeting signals for protein import (Fig. 1). Transit peptides are highly diverse in length and primary structure, and can range from 30 to 150 amino acids (Chotewutmontri *et al.*, 2017; Lee and Hwang, 2018). Although there are limited exceptions, transit peptides are typically cleaved from the preprotein upon entry of the protein into the plastid stroma (Park *et al.*, 2018). Like other organelle targeting systems, a number of cytosolic chaperone systems, including members of the heat shock protein (Hsp) 70 and Hsp90 families, are implicated in aiding transit of preproteins to the organelle surface (Fig. 1) (Kourtz and Ko, 1997; May and Soll, 2000; Qbadou *et al.*, 2006; Flores-Pérez and Jarvis, 2013; Chotewutmontri and Bruce, 2015). These chaperone complexes probably interact with the unfolded or partially folded preprotein as it is synthesized, to prevent misfolding, aggregation, or mistargeting prior to recognition by the protein import machinery. Cytosolic chaperones are common features of all protein targeting systems and were probably recruited to the chloroplast protein import system from the host cell to fulfill a general chaperone function.

The recognition of plastid preproteins at the outer envelope membrane of plastids is mediated by binding of the transit peptide to the core TOC complex, composed of Toc34, Toc159, and Toc75 (Fig. 1) (Paila *et al.*, 2015; Chotewutmontri *et al.*, 2017). Toc34 and Toc159 are membrane-bound GTPases that function as the primary import receptors at the outer membrane (Kessler and Schnell, 2009; Schleiff and Becker, 2011; Chang *et al.*, 2012). They initiate transit peptide binding to the TOC complex and control the early steps in import via their intrinsic GTPase activities, thereby constituting a checkpoint in the import reaction to ensure the fidelity of targeting (Chang *et al.*, 2017; Richardson *et al.*, 2018; Wiesemann *et al.*, 2019). Toc75 is the major component of the protein import channel at the outer membrane (Fig. 1) (Ganesan and Theg, 2019). Toc75 also binds to transit peptides, and it functions in coordination with the GTPase receptors to mediate the insertion of the transit peptide across the outer envelope membrane (Schleiff *et al.*, 2003; Kikuchi *et al.*, 2006; Chen and Li, 2007; Koenig *et al.*, 2010). Toc75 is essential in Arabidopsis (Jackson-Constan and Keegstra, 2001; Baldwin *et al.*, 2005; Hust and Gutensohn, 2006), and site-specific cross-linking demonstrates its intimate association with preproteins at all stages in import (Ma *et al.*, 1996; Richardson *et al.*, 2018).

Proteins destined for the plastid interior must traverse both the outer and inner envelope membranes (Schnell and Blobel, 1993; Kouranov *et al.*, 1998; Inoue and Akita, 2008). To achieve this, the TOC complex associates with a second transport system at the inner membrane, designated TIC, to form supercomplexes that facilitate direct translocation of the protein from the cytoplasm into the stroma (Fig. 1) (Kikuchi *et al.*, 2013; Chen and Li, 2017; Richardson *et al.*, 2018). The coordinate action of the TOC and TIC systems is facilitated by Tic236, a component that appears to form a physical link between TOC, the TIC membrane channel, and components of

the ATP-dependent molecular motor responsible for driving protein import across the two membranes (Chen *et al.*, 2018). Transit of preproteins through the intermembrane space is also facilitated by the Tic22 small chaperones (Kouranov *et al.*, 1998; Qbadou *et al.*, 2007; Kasmati *et al.*, 2013; Rudolf *et al.*, 2013).

Tic20 and Tic110 are proposed to constitute components of the TIC membrane channel at the inner membrane (Fig. 1) (Chen *et al.*, 2002; Hirabayashi *et al.*, 2011; Kasmati *et al.*, 2011; Kovacs-Bogdan *et al.*, 2011). The membrane channel activity of Tic20 has been demonstrated (Kovacs-Bogdan *et al.*, 2011), and it is in close proximity to the transit peptide as the preprotein initiates translocation across the envelope (Richardson *et al.*, 2018). Tic110 also contains a transit peptide-binding site, and this property facilitates the handoff of the protein import substrate to the chaperones to initiate ATP-dependent translocation through TOC–TIC supercomplexes (van den Wijngaard and Vredenberg, 1999; Inaba *et al.*, 2003; Chou *et al.*, 2006; Richardson *et al.*, 2018). Tic110 and the membrane-bound co-chaperone, Tic40, also form a scaffold for the binding of chloroplast stromal chaperones (Kessler and Blobel, 1996; Lubeck *et al.*, 1996; Nielsen *et al.*, 1997; Jackson *et al.*, 1998; Chou *et al.*, 2003, 2006; Inaba *et al.*, 2003, 2005; Kovacheva *et al.*, 2005; Kao *et al.*, 2012). The import-associated chaperone complex in the stroma includes members of the Hsp70 (cpHsp70), Hsp90 (Hsp90C), and Hsp100 (ClpC/Hsp93) families (Fig. 1) (Flores-Pérez and Jarvis, 2013). Although the precise role of each chaperone remains to be defined, the combination of molecular genetic, biochemical, and physiological data conclusively demonstrates that the import-associated chaperone complex acts as the ATP-dependent translocation motor for protein import across the envelope (Constan *et al.*, 2004; Kovacheva *et al.*, 2007; Su and Li, 2010; Inoue *et al.*, 2013; Liu *et al.*, 2014). Their activities also are likely to facilitate protein quality control, protein folding, or subsequent targeting events once the protein has completed import into the organelle.

Origins of plastid protein import

The origins of transit peptides remain a mystery, due in large part to their remarkable size and sequence diversity (Bruce, 2000; Lee and Hwang, 2018). However, similar extensions are absent in the corresponding ancestral cyanobacterial genes, indicating that transit peptides are probably of host origin and co-evolved with the TOC–TIC system. Regardless of the exact origin, recent studies suggest that transit peptides, particularly in vascular plants, have evolved to consist of tandemly arranged functional motifs that mediate the interactions of preproteins with various import components (Li and Teng, 2013; Lee and Hwang, 2018; Holbrook *et al.*, 2016). This hypothesis is consistent with the demonstrated interactions of transit peptides with TOC and TIC proteins at each stage in import (Richardson *et al.*, 2018), and it is one possible mechanism to facilitate sequential, unidirectional transport of the preprotein from the cytoplasm into the stroma. Furthermore, the modular arrangement of transit peptides provides the potential for evolving new or modified

molecular recognition motifs to enhance the interactions with specific import components, and thereby regulate the import of specific proteins during plastid biogenesis or plastid-type transitions (see below).

Phylogenetic analyses of the general import machinery demonstrate that they are conserved across all lineages of the plant kingdom, providing compelling evidence that the primary endosymbiotic event that gave rise to chloroplasts was a rare, if not singular, event (McFadden, 2014; Zimorski *et al.*, 2014; Garg and Gould, 2016). Toc75, the TOC channel component, is highly conserved, even amongst secondary endosymbiont lineages (Voulhox *et al.*, 2003; Inoue and Potter, 2004; Schleiff and Soll, 2005; Hsu and Inoue, 2009; Schleiff and Becker, 2011; Noinaj *et al.*, 2013). Clues as to the origin of the Toc75 channel first emerged when sequence analysis demonstrated that it belongs to the outer membrane protein (OMP) 85 superfamily of β -barrel proteins that are found exclusively in the outer membranes of Gram-negative bacteria, mitochondria, and chloroplasts (Day *et al.*, 2014; Simmerman *et al.*, 2014; O'Neil *et al.*, 2017). This family of proteins is characterized by the presence of a C-terminal β -barrel membrane domain, which typically functions in protein transport or insertion at the membrane, and a variable number of structurally related N-terminal polypeptide transport-associated (POTRA) domains, which interact with the protein substrates and accessory factors to facilitate protein transport (Paila *et al.*, 2016; O'Neil *et al.*, 2017).

The OMP85 family includes the membrane components of the β -barrel assembly machinery (BAM) and translocation and assembly module (TAM) complexes, BamA/TamA, that

participate in the transport and assembly of bacterial OMPs. On this basis, it has been proposed that Toc75 in chloroplasts was derived from an ancestral OMP85 β -barrel membrane transporter gene related to the BAM/TAM systems of the original endosymbiont (Fig. 2) (Day *et al.*, 2014; Heinz and Lithgow, 2014). In support of this hypothesis, a second TAM homolog, Tic236, was recently identified in chloroplasts. Tic236 is phylogenetically related to TamB, which links the bacterial inner membrane with BAM/TAM, thereby facilitating passage of substrates from the Sec secretion system to the BAM in the outer membrane (Chen *et al.*, 2018; Schnell, 2018). Tic236 interacts with both Toc75 and Tic110, which suggests that it co-evolved with Toc75 to maintain a link between outer and inner membranes while simultaneously acquiring new interactions that established a link between TOC and TIC channels (Fig. 2) to facilitate preprotein import across both envelope membranes simultaneously. Both Toc75 and Tic236 are closely related to proteins found in the outer membrane of the cyanobacterium, *Synechocystis* sp. PCC 6803 (Bölter *et al.*, 1998; Reumann *et al.*, 1999; Chen *et al.*, 2018). Although there is no evidence that the cyanobacterial proteins are functional orthologs of the chloroplast proteins, these observations are consistent with the proposal that the Toc75–Tic236 system arose from the duplication and/or adaptation of cyanobacterial genes.

In addition to interacting with Tic236 (Chen *et al.*, 2018), the POTRA domains of Toc75 bind transit peptides and the small intermembrane space chaperones, Tic22-III and Tic22-IV (Fig. 1) (Paila *et al.*, 2016). The POTRA domains also possess chaperone activity (Paila *et al.*, 2016; O'Neil *et al.*, 2017) and,

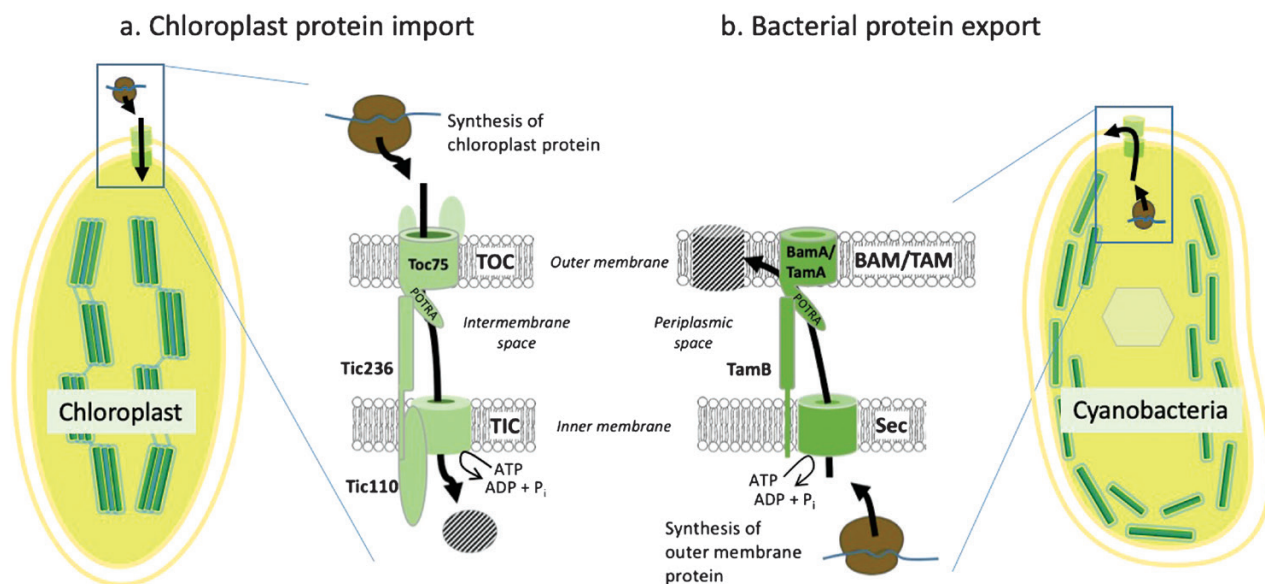


Fig. 2. Core components of the chloroplast general protein import system evolved from bacterial protein export systems. (a) Protein import is mediated by TOC and TIC complexes in the outer and inner envelope membranes of plastids, respectively. Import occurs simultaneously through TOC and TIC supercomplexes to avoid mistargeting of preproteins to the intermembrane space. Supercomplexes appear to be assembled by the binding of Tic236 to the TOC import channel, Toc75, and the core TIC component, Tic110. Tic110 is associated with the TIC import channel and the ATP-dependent import motor that drives preprotein import. (b) Tic236 and its binding partner in the TOC complex, Toc75, are related to TamB and BamA/TamA of the BAM/TAM protein export complexes from Gram-negative bacteria, respectively. Therefore, it appears that key elements of the chloroplast protein import system were derived from the bacterial protein export system during the evolutionary assimilation of the photosynthetic bacterial ancestor by a host cell during endosymbiosis. The adaptation of the chloroplast import apparatus from BAM/TAM components required the acquisition of additional TOC and TIC components to reverse the directionality of protein translocation and confer specificity for the import of nuclear-encoded plastid preproteins.

together with the Tic22 proteins, are proposed to provide a chaperone complex to facilitate preprotein transit across the intermembrane space. These activities are analogous to the role of the POTRA domains of BamA, which constitute a platform for binding of the periplasmic chaperone, SurA, to facilitate passage of the secreted substrate from the Sec machinery in the inner membrane to the BamA membrane insertase in the outer membrane (Noinaj *et al.*, 2015). Cyanobacteria also possess proteins related to the Tic22-III and Tic22-IV chaperones, but their function in bacteria is unknown (Tripp *et al.*, 2012). Nonetheless, it suggests that the existing chaperone activity of these components was adapted for protein import into chloroplasts.

The nature and origins of the TIC channel have been intensely investigated for many years. The accumulated evidence demonstrates that Tic20 plays a central role in membrane transport of preproteins at the inner membrane in chloroplasts (Fig. 1) (Ma *et al.*, 1996; Kouranov and Schnell, 1997; Teng *et al.*, 2006; Kovacs-Bogdan *et al.*, 2011; Kikuchi *et al.*, 2013; Richardson *et al.*, 2018). Tic20 and a second structurally similar protein, Tic21 (Teng *et al.*, 2006), are differentially expressed and probably have overlapping functions as central components of the import channel. Like Toc75 and Tic236, these core components of the TIC preprotein channel also appear to have been adapted from cyanobacterial genes. However, they are not derived from known bacterial protein transport systems. Proteins with ~30% sequence identity to both Tic20 and Tic21 have been identified in cyanobacteria (Reumann and Keegstra, 1999; Lv *et al.*, 2009). The Tic21-like protein from *Synechocystis* sp. PCC 6803, SynTic21, complements the phenotypes of a null mutant lacking Tic21 in Arabidopsis, demonstrating that Tic21 from plants and cyanobacteria are orthologous (Lv *et al.*, 2009). The cyanobacterial ortholog of Tic21 also possesses iron transport activity (Duy *et al.*, 2007), raising the possibility that the components of the TIC channel were adapted from existing nutrient transporters in the cyanobacterial inner membrane.

One critical adaptation during the evolution of the chloroplast TOC–TIC system was the apparent reversal in transport function relative to the bacterial export systems (Fig. 2) (Day and Theg, 2018). BAM/TAM mediate export of proteins from the periplasmic space into the outer membrane, whereas TOC–TIC functions in the opposite direction to import proteins from the cytoplasm to the stroma. The reversal was accomplished, in part, by a highly specific targeting system, consisting of the transit peptide and the corresponding transit peptide receptors, Toc34 and Toc159, which evolved to mediate delivery of the preprotein to the Toc75 channel. The TOC receptors are essential in Arabidopsis and are found in all plant lineages (Jarvis *et al.*, 1998; Bauer *et al.*, 2000; Constan *et al.*, 2004; Ivanova *et al.*, 2004; Kubis *et al.*, 2004; McFadden and van Dooren, 2004). They belong to the translation factor (TRAFAC)–related superclass of GTPases that include many cellular regulatory proteins (Leipe *et al.*, 2002), and they can be further subclassified into the GTPases activated by dimerization (GAD) subfamily (Wittinghofer and Vetter, 2011). This subfamily includes other protein targeting factors, most notably the SRP that mediates the targeting of secretory proteins to the endoplasmic reticulum (ER). Although a clear ancestral

lineage for Toc34 and Toc159 has not been defined (Reumann *et al.*, 2005), they are likely to be of host origin and evolved to take advantage of the robust and pervasive GTPase regulatory mechanism to control preprotein targeting to the TOC complex. Consistent with their inclusion in the GAD subfamily, Toc34 and Toc159 control transit peptide recognition via a cycle of receptor dimerization that regulates nucleotide exchange and hydrolysis (Oreb *et al.*, 2011; Lumme *et al.*, 2014). In the case of the TOC receptors, the GTPase activators and effectors that are commonly associated with other regulatory GTPases to control their activities appear to be replaced by the transit peptide. Binding of the transit peptide triggers changes in receptor dimerization to allow nucleotide exchange and hydrolysis (Oreb *et al.*, 2011; Wiesemann *et al.*, 2019). Inhibiting this GTPase cycle prevents stable association of the transit peptide with the TIC channel and engagement of the import-associated chaperone motor, thereby blocking protein import (Perry and Keegstra, 1994; Ma *et al.*, 1996; Kouranov and Schnell, 1997; Oreb *et al.*, 2011; Chang *et al.*, 2017; Richardson *et al.*, 2018). As such, the TOC receptors have evolved to couple transit peptide recognition with a GTPase-driven checkpoint to ensure the fidelity of protein import and initiate directional transport across the outer membrane.

The acquisition of Tic110 was a second key element in establishing the directionality of import. It constitutes a critical link between the TOC and TIC import channels and the import motor via its association with Tic236, Tic20, and the stromal chaperones (Fig. 2). Tic110 also interacts with transit peptides and Tic40, a stromal co-chaperone associated with the inner membrane (Chou *et al.*, 2003, 2006). Tic110 (Tsai *et al.*, 2013) and Tic40 (Chou *et al.*, 2003; Kao *et al.*, 2012) contain conserved sequence motifs that mediate their interactions with stromal chaperones and stimulate chaperone ATP hydrolysis. By acting as a scaffold for chaperone organization, Tic110 and Tic40 tether the ATP-dependent preprotein binding and release cycle of the chaperones to the site of import, thereby providing the unidirectional driving force for translocation of preproteins from the cytoplasm to the stroma. Consistent with these roles, phylogenetic analysis demonstrates Tic110's and Tic40's universal presence in the green lineage (McFadden, 2014; Zimorski *et al.*, 2014; Garg and Gould, 2016). Like the TOC receptors, evolution probably took advantage of ubiquitous structural and functional motifs to assemble and adapt Tic110 and Tic40 for preprotein and chaperone binding.

It is estimated that the protein import motor requires the hydrolysis of 650 ATP molecules per polypeptide to import the average preprotein (Shi and Theg, 2013). Although the relative contributions of each chaperone have not been fully defined, it is now generally accepted that the ATP-dependent import motor is composed of the stromal cpHsp70, Hsp90C, and Hsp93/ClpC chaperones (Kessler and Blobel, 1996; Akita *et al.*, 1997; Nielsen *et al.*, 1997; Shi and Theg, 2010; Su and Li, 2010; Inoue *et al.*, 2013). Each of the chloroplast chaperones plays multiple roles in protein homeostasis within the organelle, and therefore they are not specialized to function in protein import alone. Albeit somewhat more complex, the import-associated motor is similar to the Hsp70-based motors that function in protein import or membrane translocation

in mitochondria and the ER (Flores-Pérez and Jarvis, 2013). Thus, a similar mechanism has been adapted in numerous protein import systems to catalyze membrane transport.

Recently, complexes containing the plastid-encoded hypothetical chloroplast ORF (Ycf) 1 (recently named Tic214) and Ycf2 proteins of the inner envelope have been proposed to function as components of the TIC channel and import motor, respectively, in coordination with Tic20 (Kikuchi *et al.*, 2013, 2018). Components of the Tic214 and Ycf2 complexes are absent from major algal and plant lineages (Glucophyta, Rhodophyta, the Poaceae, and some other dicot species) (Huang *et al.*, 2013; Kikuchi *et al.*, 2013; de Vries *et al.*, 2015), and an evolutionary survey of Ycf1/Tic214 also demonstrates a high degree of structural variability across species, a trait uncharacteristic of the high degree of conservation in confirmed TOC and TIC components (de Vries *et al.*, 2015). Furthermore, studies from other groups demonstrate limited reduction in protein import activity when Tic214 complex components were depleted in *Arabidopsis* (Köhler *et al.*, 2016; Agne *et al.*, 2017; Bölter and Soll, 2017). Therefore, the precise roles of the Tic214 and Ycf2 complexes in protein import remain to be clarified, and more research is necessary to determine how these new components can be integrated into current, established models of protein import. As discussed below, there is growing evidence that the protein import apparatus plays a direct role in the regulation of plastid biogenesis and homeostasis. With this in mind, it will be important that investigators look beyond the constraints of current models of the import mechanism and consider alternative functions for these new components, including TIC assembly, protein quality control, and TOC–TIC regulation, or as mediators of substrate- or species-specific import (Paila *et al.*, 2015; Sjuts *et al.*, 2017; Bölter, 2018).

Regulation of chloroplast protein import during development and stress

Expansion and functional diversification of TOC complexes

The evolution of land plants resulted in expansion of the plastid family beyond chloroplasts to fulfill specialized metabolic, physiological, and signaling activities in distinct cell types, organs, and tissues (de Vries *et al.*, 2016). More than a dozen plastid types are now recognized, and include proplastids, the undifferentiated precursors for all plastid types found in meristematic tissue; starch-storing amyloplasts, which are also important in gravitropism; pigment-containing chromoplasts such as those found in tomato fruit; and gerontoplasts, which function in nutrient re-mobilization in senescing tissues (Kiss *et al.*, 1989; Lopez-Juez and Pyke, 2005; Jarvis and Lopez-Juez, 2013; Pinard and Mizrachi, 2018). All of these plastid types have unique proteomes, resulting from developmentally or environmentally triggered changes in gene expression. For example, as much as one-third of the cellular transcriptome in seedlings changes in response to light during photomorphogenesis (Ma *et al.*, 2001; Tepperman *et al.*, 2001). Many of the

transcriptome changes lead to the synthesis of plastid proteins that are critical for the transition of proplastids and etioplasts to photosynthetically competent chloroplasts. It is now recognized that the TOC–TIC general protein import system has also diversified during evolution to accommodate the changes in the organelle proteome that are required to maintain basic organelle activities while transitioning to perform specialized functions (Bauer *et al.*, 2000; Kubis *et al.*, 2003, 2004; Inoue *et al.*, 2010). Furthermore, additional evidence indicates that the import apparatus is directly regulated as part of key transitions in plastid type and function (Ling *et al.*, 2012, 2019; Chu and Li, 2018).

The TOC GTPase receptor families have expanded and diversified in parallel with the expansion of the plastid family in the green lineage (Reumann *et al.*, 2005; Kalanon and McFadden, 2008). Toc159 and Toc34 are encoded by small gene families in vascular plants, and the receptor variants exhibit distinct spatial and temporal expression patterns. In *Arabidopsis*, null mutants in different Toc159 or Toc34 isoforms impact plastid biogenesis in distinct ways (Bauer *et al.*, 2000; Ivanova *et al.*, 2004; Kubis *et al.*, 2004; Inoue *et al.*, 2010; Infanger *et al.*, 2011; Dutta *et al.*, 2014). These receptor isoforms assemble with a single Toc75 protein to form TOC complexes that mediate the selective import of subsets of precursor proteins (Fig. 3a). For example, atToc159 and atToc33 are the major isoforms in green tissues in *Arabidopsis*, and both play central roles in the import of photosynthetic proteins and other processes required for chloroplast biogenesis. AtToc132/120 and atToc34, other Toc159 and Toc34 family members, respectively, are expressed at similar levels in most tissues and appear to be essential for the maintenance of metabolic functions that are common to different plastid types (Fig. 3a) (Bauer *et al.*, 2000; Kubis *et al.*, 2003, 2004; Inoue *et al.*, 2010; Bischof *et al.*, 2011). These observations provided the first evidence that the general import apparatus plays a direct role in controlling the changes in the plastid proteome that are required for plastid-type specific functions. The co-evolution of distinct transit peptide classes and import complexes with correspondingly distinct specificities was probably a key adaptation that enabled the proteome changes necessary for functional or plastid-type transitions while maintaining organelle homeostasis.

At least three distinct functional classes of transit peptides have also been identified that mediate preferential import into plastids at different developmental stages (Fig. 3a) (Teng *et al.*, 2012). It has been proposed that the modular organization of transit peptides provides the platform for altering recognition by specific import components (e.g. distinct TOC receptors), while maintaining interactions with invariant import components (e.g. Toc75 or Tic110) (Li and Teng, 2013). In doing so, the import of functionally related subclasses of preproteins can be regulated in response to developmental or physiological signals that require changes in plastid function.

Regulation of protein import by the ubiquitin–proteasome system

The changes in the profiles of imported proteins during plastid-type transitions or organelle responses to physiological

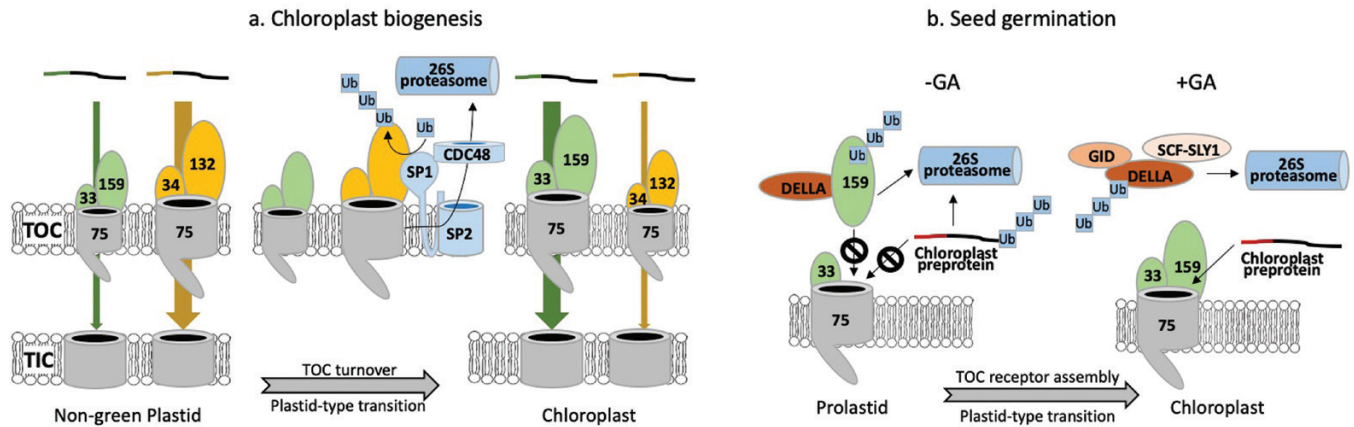


Fig. 3. Regulation of the plastid general import machinery by the ubiquitin–proteasome system (UPS) during developmental transitions. Distinct TOC complexes mediate the import of specific classes (Groups 1 and 2) of nuclear-encoded preproteins into plastids. The specificity of the import complexes is determined by the Toc34–Toc159 family of transit peptide receptors. The relative abundance of distinct TOCs is regulated by the UPS and is required to balance the capacity of distinct import pathways with the changes in gene expression that accompany proteome remodeling during plastid-type transitions. (a) During the transition from non-green plastids to chloroplasts, the abundance of TOC complexes containing Toc132/Toc33 receptors (orange) relative to Toc159/Toc33 receptors (green) is altered to accommodate the import of proteins required for photosynthesis and the transition from chemoautotrophic to photoautotrophic metabolism. UPS-mediated turnover of TOC complexes involves a RING-type E3 ubiquitin ligase (SP1) that polyubiquitinates TOC components. The ubiquitinated TOC components appear to be extracted from the outer membrane and delivered to the 26S proteasome for degradation by the combined activities of the SP2 β -barrel channel protein and the cytosolic Cdc48 AAA+ ATPase. (b) Gibberellic acid (GA) plays an important part in preventing the premature biogenesis of chloroplasts during seed germination. The DELLA transcriptional regulators accumulate in the absence of GA and suppress the transcription of genes associated with germination, including genes required for chloroplast biogenesis. DELLAs also bind to the cytosolic form of the Toc159 protein import receptor, thereby promoting receptor degradation by the UPS and preventing the formation of TOC complexes required for the import and assembly of the photosynthetic machinery. A GA-regulated quality control system also targets cytosolic chloroplast preproteins for UPS degradation to prevent the accumulation of toxic preproteins in the cytosol. During germination, increased GA promotes UPS-mediated degradation of DELLAs via the GA–Gibberellin insensitive dwarf1 (GID) complex, the F-box protein SLY1, and the SCF E3 ligase, thereby inducing the expression of genes required for assembly of the photosynthetic machinery, while simultaneously allowing Toc159 to assemble with other TOC components to form complexes required for chloroplast biogenesis.

events require rapid modifications to the absolute and relative levels of specific TOC complexes. Two components involved in turnover of TOC complexes were discovered in a screen for suppressors of the pale phenotype of the *atToc33* knockout mutant, *ppi1*. Suppressor of *ppi1 locus 1* (SP1) is an E3 ubiquitin ligase that localizes to the chloroplast envelope and promotes turnover of *atToc75*, *atToc159*, and *atToc33* via the ubiquitin–proteasome system (UPS) (Ling *et al.*, 2012). The second component, SP2, is a β -barrel, OMP85 family member that is proposed to serve as a channel (retrotranslocon) for extraction of ubiquitinated TOC proteins from the membrane. SP1 and SP2 appear to associate with a cell division cycle protein (Cdc) 48 AAA+ ATPase in the cytosol, which is proposed to provide the pulling force for TOC extraction through SP2. Together, these components comprise a system hypothesized to function in a manner analogous to that which exists as part of the ER-associated degradation (ERAD) system, termed CHLORAD (Fig. 3a) (Ling *et al.*, 2019). In fact, Cdc48 is a common component of the SP1–SP2 and ERAD systems. Knockout of SP1 or SP2 has no obvious growth defects under normal growth conditions; however, the *sp1* mutant shows defects in chloroplast biogenesis during de-etiolation, and both *sp1* and *sp2* mutants attenuate photosynthetic decreases in mature leaves following premature dark treatment, mimicking leaf senescence. Conversely, SP1 or SP2 overexpression has an enhanced senescence phenotype, and SP1 overexpression shows enhanced de-etiolation (Ling *et al.*, 2012, 2019). These observations are consistent with an important role for SP1 and

SP2 during developmental transitions that involve plastid differentiation and/or changes in photosynthetic capacity (Fig. 3a) (Ling *et al.*, 2012; Ling and Jarvis, 2015).

SP1 is a member of the expansive family of really interesting new gene (RING) E3 ubiquitin ligases in plants (Mazzucotelli *et al.*, 2006). It was recently shown to also localize to peroxisomes and mitochondria in addition to chloroplasts (Pan *et al.*, 2016; Pan and Hu, 2018). At peroxisomes, it is proposed that SP1 participates in turnover of the peroxisomal protein import machinery analogous to its role in chloroplasts, and negatively regulates peroxisome function (Pan *et al.*, 2016). The co-localization of SP1 to chloroplasts, peroxisomes, and mitochondria may suggest the evolution of a common mechanism to coordinate the biogenesis of these three organelles by modulating protein import during developmental processes such as greening. This would serve as a mechanism to promote and regulate their coordinate metabolic functions, for example in photosynthesis, photorespiration, and during germination. Further work is needed to establish the targeting mechanism of SP1 to multiple organelles and its physiological relevance (Ling *et al.*, 2017). Nonetheless, this observation may hint at coordinated regulation of the proteomes of chloroplasts and peroxisomes (and possibly mitochondria) during development by modulation of the levels and specificity of their protein import systems.

The ability to control plastid differentiation and chloroplast development during seedling establishment was also a key component of the adaptation of vascular plants to terrestrial life.

Seeds are specially equipped to protect embryos from the harsh conditions of life on land, such as high light and an arid atmosphere, relative to the aquatic habitat of their ancestors (de Vries *et al.*, 2016). In *Arabidopsis* (and most other dicots), proplastids are present in all cotyledon cells and develop into chloroplasts upon illumination, which involves extensive alterations of cellular and plastid proteomes. Hormone signaling pathways including gibberellic acid (GA), brassinosteroids (BRs), abscisic acid (ABA), and auxin also control chloroplast biogenesis during germination and early seedling development (reviewed in Pogson *et al.*, 2015; Nee *et al.*, 2017), and autophagy and protein turnover via the UPS contribute to remodeling of the cellular (and chloroplast) proteome during photomorphogenesis (Woodson, 2016; Aguilar-Hernandez *et al.*, 2017).

It was recently reported that GA signaling regulates turnover of the major protein import receptor in *Arabidopsis*, atToc159, through the DELLA transcriptional regulators during seed germination (Fig. 3b) (Shanmugabalaji *et al.*, 2018). GA promotes germination and chloroplast development by inducing degradation of DELLAs, which in turn induces expression of photosynthetic and chloroplast biogenesis genes. In the absence of GA, DELLAs accumulate and prevent this transcriptional response, suppressing chloroplast development in the dark to prevent premature greening and photooxidation caused by accumulation of chloroplast precursors (Cheminant *et al.*, 2011; Pogson *et al.*, 2015). In addition to transcriptional suppression of chloroplast biogenesis, the chloroplast protein import receptor atToc159 is degraded under low GA in a DELLA-dependent manner, and is stabilized in the presence of GA (Shanmugabalaji *et al.*, 2018). Notably, atToc33 is also degraded under low GA, whereas atToc132 and atToc75 are not (Shanmugabalaji *et al.*, 2018). This is consistent with the role of the atToc159 and atToc33 receptors in the import of a subset of preproteins that are critical for chloroplast biogenesis and assembly of the photosynthetic apparatus (Bauer *et al.*, 2000; Kubis *et al.*, 2003, 2004; Ivanova *et al.*, 2004; Inoue *et al.*, 2010). Low GA also promotes ubiquitination of the precursor to the Rubisco small subunit and reduces its accumulation in the cytosol, thereby preventing the toxic accumulation of the preprotein in the cytoplasm in the absence of a functional TOC import complex (Fig. 3b). atToc159 degradation in response to low GA appears to be independent of the SP1 E3 ligase, whereas Toc75 and atToc33 turnover is dependent upon the presence of SP1 (Shanmugabalaji *et al.*, 2018). This suggests a novel mode of regulation for chloroplast biogenesis during seed dormancy and germination, in which the composition of the import apparatus is tightly regulated, and chloroplast precursor protein accumulation is minimized by a GA signaling mechanism to prevent premature greening. While this mechanism was studied only in germinating seeds, it is possible that similar mechanisms exist to regulate import during other key plastid developmental transitions.

Biotic and abiotic stress, including light, salt, drought, and osmotic stress, result in high levels of photooxidative damage to chloroplast proteins, and protein turnover is an important means by which plants contend with oxidative damage (Li *et al.*, 2009; Nelson *et al.*, 2014; Jarvi *et al.*, 2015; Woodson, 2016; Otegui, 2018). Consequently, the import of photosynthetic

proteins and balancing the stoichiometry of nuclear- and chloroplast-encoded subunits of the multisubunit photosynthetic apparatus is critical for maintenance and repair of the photosynthetic apparatus under stress. This was recently underscored by the finding that loss or overexpression of SP1 or SP2 renders *Arabidopsis* plants hypersensitive or resistant to salt, osmotic, and oxidative stresses, respectively. These observations were linked to the ability of SP1 to control the levels of specific TOC components, and thereby the import of photosynthetic proteins, which ultimately controls the levels of photooxidation and reactive oxygen species (ROS) production (Ling and Jarvis, 2015; Ling *et al.*, 2019). It has also been proposed that manipulation of the SP1–SP2 protein import control system could be exploited by pathogens to minimize host responses to infection (Sowden *et al.*, 2018). It is possible that modulation of protein import into peroxisomes (and mitochondria) additionally contributes to the stress-tolerant phenotype of the SP1 overexpression plants, since both organelles are also involved in ROS generation and signaling (Hu *et al.*, 2012).

The TOC receptors are also phosphorylated, although the physiological significance of this post-translational modification is still somewhat unclear (Fulgosi and Soll, 2002; Aronsson *et al.*, 2006; Oreb *et al.*, 2008; Agne *et al.*, 2010; Zufferey *et al.*, 2017). The atToc159 receptors are phosphorylated in an ABA-dependent manner; atToc159 by the kinase SnRK2, and atToc132 and Toc120 in an SnRK2-independent manner (Wang *et al.*, 2013). The chloroplast outer envelope kinase, KOC1, also phosphorylates Toc159, and *koc1* mutants show defects in chloroplast protein import, demonstrating that phosphorylation by this kinase is an important regulator of import (Zufferey *et al.*, 2017). It has also been proposed that phosphorylation regulates the dimerization capabilities of the Toc GTPase receptors, and negatively regulates their interaction with preproteins, which may also be phosphorylated (Fulgosi and Soll, 2002; Oreb *et al.*, 2008). Although direct evidence is lacking (Aronsson *et al.*, 2006), these observations suggest that phosphorylation plays a role in regulating the TOC complex, and one possibility is that phosphorylation is tied to UPS-mediated degradation of TOC components.

Coordinate regulation of protein import with plastid–nucleus communication

Protein import and chloroplast to nucleus retrograde signaling

Chloroplasts have also evolved important roles in sensing physiological and environmental changes as part of the organelle–nucleus communication networks that were critical for the integration of host and endosymbiont. Complex chloroplast to nucleus signaling pathways have been identified that relay the status of the chloroplast to the nucleus to control expression of nuclear-encoded chloroplast genes. The most well studied signals involve tetrapyrroles produced in the chloroplast, plastid gene expression, ROS, and chloroplast metabolites (Chi *et al.*, 2015; Pogson *et al.*, 2015; Chan *et al.*, 2016; Kleine and Leister, 2016). A chloroplast unfolded protein response was

also recently shown to trigger a nuclear response upon disruption of protein homeostasis within the chloroplast. Chloroplast signals result in the up-regulation of nuclear-encoded chloroplast chaperones (Llamas *et al.*, 2017).

Perturbance of chloroplast protein import also elicits a nuclear response. Plants that lack the major chloroplast protein import receptor Toc159 (*plastid protein import 2*; *ppi2*) have an albino phenotype and are defective in the import of several photosynthetic proteins but accumulate many other non-photosynthetic proteins normally (Bauer *et al.*, 2000; Bischof *et al.*, 2011). Expression of nuclear-encoded photosynthetic genes is down-regulated in *ppi2* (Bauer *et al.*, 2000; Kakizaki *et al.*, 2009; Lee *et al.*, 2009), suggesting that a plastid to nucleus signaling pathway exists to attenuate photosynthetic gene expression in the absence of Toc159. This signal does not appear to involve Mg-Protoporphyrin IX (Mg-ProtoIX), a chlorophyll intermediate that is proposed to be a chloroplast retrograde signaling molecule, as *ppi2* does not accumulate Mg-ProtoIX (Kakizaki *et al.*, 2009; Chi *et al.*, 2015; Chan *et al.*, 2016; Kleine and Leister, 2016). Plastid to nucleus signaling in *ppi2* does involve GUN1, a plastid protein known to be involved in multiple plastid–nucleus signaling pathways, and the transcription factor Golden-like 1 (GLK1), a key regulator of chloroplast biogenesis (Fitter *et al.*, 2002; Kakizaki *et al.*, 2009). There appear to be (at least) two plastid signals that dampen the GLK1 transcriptional response in *ppi2*; one that down-regulates transcription of *GLK1*, and the other that results in degradation of GLK1 via the UPS in a GUN1-independent mechanism (Kakizaki *et al.*, 2009; Tokumaru *et al.*, 2017). There are still many remaining questions about how the nucleus senses defects in protein import, and whether this response is important during normal plant growth and development. Nonetheless, these observations point toward a system for sensing disruptions in protein import that is distinct from the known pathways that monitor chloroplast damage. The identification of key signaling molecules/proteins specific to protein import stress will be important for understanding protein import regulation during development and environmental stress.

Inhibition of protein import and the cytosolic unfolded protein response

When chloroplast protein import is down-regulated or perturbed, accumulating chloroplast precursors in the cytosol can have toxic effects on the cell. In addition to plastid signals that down-regulate nuclear gene expression of photosynthetic proteins, the cell also has at least one known mechanism for removing accumulating chloroplast precursors from the cytosol. In *ppi2*, chloroplast precursors that are unable to be imported efficiently due to the absence of Toc159 are degraded in the cytosol via the UPS, which involves the activity of the E3 ligase C-terminus of Hsp70-interacting protein (CHIP) (Lee *et al.*, 2009). The cytosolic chaperone Hsp70-4, which is one of at least five cytosolic Hsp70 chaperones in Arabidopsis (Lin *et al.*, 2001; Sung *et al.*, 2001; Lee *et al.*, 2009), is highly up-regulated in *ppi2* and binds to the transit peptide of cytosolic chloroplast precursor proteins. Hsp70-4 recruits CHIP, which results in ubiquitination of accumulating precursors that

are subsequently degraded by the proteasome (Lee *et al.*, 2009). The inability of Hsp70-deficient plants to remove accumulating precursors leads to cytotoxic effects that interfere with normal growth and development of plants, highlighting the importance of this proteotoxic stress response in plant growth and development (Lee *et al.*, 2009). In tomato, Hsp70 and Hsp90 are also known to be part of a general response to accumulating chloroplast precursors (Tillmann *et al.*, 2015).

In Arabidopsis, similar to in non-plant species, components of the heat shock response are important for mitigating the effects of protein unfolding in the cytosol (Sugio *et al.*, 2009; Lin *et al.*, 2018). In response to heat stress, expression of Hsps is induced by a network of transcription factors called heat shock factors (HSFs). In non-plant species such as yeast and *Drosophila*, a single HSF regulates heat shock response genes, and in mammals the HSF gene family is limited to four members (Akerfelt *et al.*, 2010). However, Arabidopsis (and other plant species) has an expanded repertoire of HSF genes, forming a gene regulatory network that is involved in the response to various biotic and abiotic stresses including pathogen, salinity, drought, and cold stress (Swindell *et al.*, 2007; von Koskull-Doring *et al.*, 2007; Guo *et al.*, 2016). In Arabidopsis, part of this network mediates a general cytoplasmic unfolded protein response that also appears to be up-regulated when protein import is impaired (Lee *et al.*, 2009; Sugio *et al.*, 2009; Gladman *et al.*, 2016; Lin *et al.*, 2018). These observations fit nicely with the recently shown GA-dependent degradation of chloroplast precursors in the cytosol during seed germination via the UPS, and are consistent with a carefully regulated system to prevent premature accumulation of chloroplast preproteins in the cytosol, which can lead to cellular toxicity.

Conclusions

The evolution of the TOC–TIC general import apparatus was essential to the integration of host and bacterial functions that accompanied endosymbiosis and the establishment of the plant kingdom. The evolution of core TOC–TIC components from bacterial protein export systems and the addition of novel components to confer directionality on targeting is a remarkable example of molecular adaptation to provide a novel function that was required for the assimilation of the new organelle. For many years, protein import was viewed simply as a basic protein trafficking pathway. We now know that the general import system plays a dynamic role in controlling and responding to changes in the flux and profiles of nuclear-encoded proteins that are required for plastid-type transitions during development and to control the flux of import in response to stress. The emergence of the import machinery as a site of regulation opens up a new phase in studies of the TOC–TIC machinery that shifts the focus from the basic mechanism to understanding how the regulation of import contributes to organelle homeostasis, developmental transitions, and plastid–nucleus signaling. Undoubtedly, recent discoveries, including the role of the UPS system in regulating import in response to developmental and hormonal signals, are only the tip of the iceberg in this new chapter of understanding the contributions of the TOC–TIC general import machinery in plastid biogenesis and function.

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