

Chloroplast proteostasis: import, sorting, ubiquitination and proteolysis

Yi Sun and R. Paul Jarvis

Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, UK;

email: paul.jarvis@plants.ox.ac.uk

Abstract

Chloroplasts are the defining organelles of plants with responsibility for photosynthesis and other vital functions. To deliver these functions they possess a complex proteome comprising thousands of largely nucleus-encoded proteins. Composition of the proteome is controlled by diverse processes affecting protein translocation and degradation – our focus here. Most chloroplast proteins are imported from the cytosol via multiprotein translocons in the outer and inner envelope membranes (TOC and TIC, respectively), or via one of several non-canonical pathways; and then sorted by different systems to organellar sub-compartments. Chloroplast proteolysis is equally complex, involving the concerted action of internal proteases of prokaryotic origin and the nucleocytoplasmic ubiquitin-proteasome system (UPS). The UPS degrades unimported proteins in the cytosol, and chloroplast-resident proteins via chloroplast-associated protein degradation (CHLORAD). The latter targets the TOC apparatus to regulate protein import, as well as numerous internal proteins directly, to reconfigure chloroplast functions in response to developmental and environmental signals.

Keywords

Chloroplast; plastid; proteasome; protein translocation; proteolysis; ubiquitin

1. INTRODUCTION

Plastids, their evolution, and protein import

Plastids are a group of related organelles that exist extensively throughout plants and a variety of algae (44). Among them, chloroplasts in the green tissues of plants attract most attention and are best studied, due to their well-known ability to photosynthetically convert the energy of light into chemical bond energy. Besides, chloroplasts also carry out the biosynthesis of many essential primary and secondary metabolites (44). Other plastid types are widely distributed in non-green plant tissues; these include amyloplasts, which are packed with starch and play important roles in energy storage in seeds, tubers, and roots, as well as in plant gravitropism, and chromoplasts, which are rich in carotenoid pigments and act as attractants in flowers and fruits (44).

One remarkable feature of plastids is their dynamism in relation to morphology and function. In response to developmental or environmental cues, the different plastid types can interconvert and such conversions play an important role in plant development; for example, during fruit ripening (when chloroplasts change into chromoplasts) and leaf senescence (when chloroplasts change to gerontoplasts) (44). Plastids are also well known for their ability to move and redistribute inside the cell, which is a critical response enabling them to deal with the environmental changes. For example, movements of chloroplasts and amyloplasts function in strong-light avoidance and gravity sensing, respectively (44).

Plastids are endosymbiotically derived organelles, and consequently contain a functional genome (the plastome) interpreted by eubacterial/phage-type transcription and translation machineries, and possess a double-membrane envelope (44). Over the course of evolution, roughly 98% of the endosymbiont's protein-coding sequences were either lost or relocated to the nuclear genome by

the process of endosymbiotic gene transfer, and only approximately 100 genes are retained in plastids (44; 112). Thus, most plastid proteins must be imported from the surrounding cytoplasm. Because all plastids within a particular organism contain the same limited complement of genes, it is the imported proteins that define the developmental fate and functions of each organelle (44).

For chloroplasts, over 90% of the 3000 or so organellar proteins are encoded in the nucleus and synthesized by cytosolic ribosomes. Most of these proteins are made as precursors (i.e., pre-proteins), having a cleavable targeting sequence at the N-terminal end called a transit peptide. The precursors are imported into chloroplasts through the interaction of the transit peptide with translocons located in the outer and inner envelope membranes called TOC and TIC (translocon at the outer/inner envelope membrane of chloroplasts), respectively (18; 22; 24; 42; 94; 109). Upon emergence from the TIC translocon, the transit peptide is cleaved off by SPP (stromal processing peptidase) (137), enabling final folding and assembly of the protein in the stroma, or onward routing to the inner envelope membrane (IEM) (73; 74; 141) or the thylakoids (15; 121) (Figure 1).

Interestingly, removable N-terminal targeting peptides also play a role in the import of nucleus-encoded proteins into photosynthetic organelles of more recent evolutionary origin, termed chromatophores, in the amoeba *Paulinella chromatophora*. The targeting of such proteins is suggested to involve the endomembrane system (101).

Proteolytic regulation of the plastid proteome

It is well established that the plastid proteome can vary considerably according to the developmental stage and environmental signals or stresses (44). Thus, it stands to reason that protein homeostasis (proteostasis) systems are highly developed in plastids, providing the regulation needed to maintain an optimally functioning organelle proteome (83). Turnover systems affecting plastid-resident proteins fall into three major categories. First, intrinsic proteases inherited from the bacterial ancestors of plastids perform quality control and maintenance functions (99; 140). Second, as has emerged more recently, the nucleocytoplasmic ubiquitin-proteasome system (UPS) plays an important role in regulating the plastid proteome (77; 99; 140). Third, under conditions of stress or senescence, the entire plastid, or a part of it, may be degraded by autophagy through its delivery to the plant vacuole (41; 102). Despite its undoubted importance, autophagy is beyond the scope of the review, and so readers are referred to the following articles for more detailed information (41; 102).

2. CHLOROPLAST PROTEIN IMPORT

2.1 Different protein targeting pathways

2.1.1 The canonical, transit peptide-mediated import route

N-terminal targeting signals are commonly used in both prokaryotes and eukaryotes to direct proteins from their site of synthesis towards their destination of function; such destinations include the bacterial membranes and, in eukaryotes, the endomembrane system, mitochondria and chloroplasts (117). The existence of such a targeting signal (or transit peptide, TP) in chloroplast precursors was first demonstrated in studies on the import of Rubisco small subunit (RbcS) (42). More recent analyses indicated that the selective retention of particular transit peptide motifs has occurred during evolution to enable the tissue-specific regulation of plastid protein import (19).

2.1.2 Envelope and non-canonical targeting pathways

Apart from the canonical, TP-mediated TOC-TIC import route, there are several other chloroplast targeting pathways that are less well understood (Figure 1, Table 1). Proteins of the outer envelope membrane (OEM) typically do not possess a TP, with targeting information instead residing within a transmembrane domain (56). Cytosolic sorting factors AKR2 (ankyrin repeat-containing protein 2) and Hsp17.8 (heat shock protein 17.8) guide such proteins to the organellar surface, while integration may require the main TOC channel formed by the protein Toc75 (TOC component of 75 kDa) (11; 55; 138). An interesting exception is the Toc75 protein itself, which possesses a bipartite signal that comprises a standard transit peptide adjacent to an intraorganellar targeting peptide that enables translocon disengagement and membrane integration (22; 40). It has recently emerged that other β -barrel proteins (e.g., OEP80 [outer envelope protein, 80 kDa], a paralogue of Toc75) may possess a TP that is necessary for chloroplast localization, while those that do not may be targeted by their penultimate β -barrel strand via the TOC apparatus (21; 60). A relatively small number of proteins of the chloroplast interior have intrinsic targeting signals and may be targeted in TOC-independent fashion (7; 92; 93), while a pathway to the plastids through the endomembrane system also exists (57; 97; 143).

2.2 Protein translocation via the TOC-TIC apparatus

2.2.1 Protein targeting to the chloroplast surface

Transit peptides are remarkably heterogeneous in length and sequence (65). This may reflect their need to accommodate various domains for interaction with sorting factors, translocon subunits, and chaperones in the cytosol, envelope membranes, and stroma (65), or with different plastid types (70). Cytosolic chaperones facilitate the navigation of pre-proteins to the organelle and maintain an unfolded conformation suitable for import (112). Two cytosolic chaperone systems are proposed to guide chloroplast pre-proteins, though their mechanistic details and physiological significance require clarification. In the first, Hsp90 cooperates with Hop (Hsp70/Hsp90-organizing protein) and the immunophilin FKBP73 to deliver pre-proteins to the OEM, docking at Toc64/OEP64 (28; 106); however, such delivery to Toc64/OEP64 is not strictly required for protein import (8; 36). In the second, Hsp70 cooperates with an undefined 14-3-3 protein to bind phosphorylated transit peptides and deliver them to translocon complexes (89); however, mutations within the 14-3-3 binding site of several pre-proteins did not impair their chloroplast targeting (96).

It is well established that the endoplasmic reticulum (ER) and mitochondria both co-translationally import polypeptides from ribosomes bound at their outer surfaces (20; 146). Similarly, recent studies in the unicellular alga *Chlamydomonas* suggest that a domain of the chloroplast outer envelope membrane is bound by ribosomes translating mRNAs for chloroplast pre-proteins (129; 139) (Figure 1, Table 1).

2.2.2 Translocation at the outer envelope membrane

The core TOC complex is formed by the proteins Toc159, Toc75 and Toc34 (Figure 2). Using artificial lipid vesicles containing these three components, it was shown that a reconstituted TOC complex indeed has the ability to bind precursors and drive their translocation through the membrane (115). The Toc33 and Toc159 components are pre-protein receptors, possessing cytosol-projecting guanosine triphosphatase (GTPase) domains that bind TPs specifically (44; 51; 118). In land plants, both receptors are encoded by gene families and so exist in multiple isoforms (the associated nomenclature is based on their molecular masses in kilodaltons) (49). In *Arabidopsis*, they are named Toc159, Toc132, Toc120 and Toc90 (Toc159 family), and Toc33 and Toc34 (Toc34 family), with different members having differing client specificity (13; 42; 43). Members of the Toc34 family have a relatively simple architecture comprising an N-terminal GTPase domain and a single C-

terminal membrane-spanning helix (42; 62; 131). Members of the Toc159 family possess a homologous GTPase domain (13), located centrally, and a much larger C-terminal membrane-anchoring domain (42). Recent advances in structural prediction reveal this domain to be a 14-stranded β -barrel, suggesting that β -barrel switching may be a feature of the chloroplast import mechanism (47). Toc159 isoforms typically also have an N-terminal, intrinsically disordered acidic domain, which may impart TP recognition specificity (3; 39).

While the TOC receptors are conserved among green lineages, including plants and green algae (94; 108), a recent study indicated that in Rhodophyta (red algae), a class of GTP-binding proteins distinct from the Toc34/159 family participates in pre-protein targeting to chloroplasts, by recognizing TPs. This suggests that Rhodophyta possess a distinct pre-protein targeting mechanism (12).

Pre-proteins are translocated across the OEM through a channel formed by the β -barrel domain of Toc75, comprising 16 β -strands. Toc75 belongs to the bacterially-descended Omp85 protein superfamily (22). A characteristic feature of Omp85 family members is the presence of a soluble N-terminal POTRA (polypeptide transport-associated) domain (61), which in the case of Toc75 extends into the intermembrane space (IMS) and performs a proposed chaperone-like role (100; 105). The core TOC complex particle was investigated by electron microscopy and estimated to have a height of 10-12 nm and a diameter of 13 nm (116). The maximum pore size of the TOC-TIC system is likely to be 30-35 Å, which is larger than those of the mitochondrial and bacterial protein translocons but smaller than that of the peroxisomal system (31; 32). The stoichiometry of the TOC core complex components (Toc34:Toc75:Toc159) was estimated to be 4-5:4:1 (116) or 3:3:1 (54), respectively, and so the channel may be formed by multiple copies of the Toc75 protein potentially in cooperation with the predicted β -strands of Toc159 (32).

Initial interaction of the TP with the GTPase domains of Toc159 and Toc34 is transient and energy independent (87), potentially allowing for rapid and sequential interaction with pre-proteins (110). Later, the TP interacts with the POTRA domain of Toc75 and the soluble IMS protein Tic22, before guanosine 5'-triphosphate (GTP) hydrolysis (110). While GTP hydrolysis at both receptors is not necessary for protein import in vivo (4; 9), it is required for successful protein translocation in vitro (110). The TP may bind simultaneously to Toc34 and Toc159, as each preferentially binds to a distinct region of the peptide (147). A bound TP may then encourage Toc34-Toc159 heterodimer formation, as well as GTP hydrolysis (86), leading to an activated translocon conformation through which the pre-protein can pass.

2.2.3 Translocation at the intermembrane space

In the IMS, Tic22 is suggested to act as a chaperone and to facilitate pre-protein delivery from TOC to TIC (136). In addition, the IEM protein Tic236 was identified as part of a 1.25 MDa TOC-TIC supercomplex (16), and suggested to provide a physical link between the TOC and TIC complexes; this idea was based on its anchorage in the IEM, where it associates with Tic20, and its interaction with the POTRA domain of Toc75 (16). In maize, the Tic236 orthologue is termed DEK5 (defective kernel 5), and was shown to have functions in envelope biogenesis (151). It was suggested to mediate the insertion of β -barrel proteins involved in protein import and metabolite transport, in accordance with the fact that it shares homology with the bacterial TamB protein (151). A role for Tic236 in protein import was proposed based on the observation that Arabidopsis *tic236* mutant chloroplasts show reduced protein import but no change in the abundance of TOC proteins (16). However, maize *dek5* mutants displayed a reduction in TOC protein abundance, and thus the impact on protein import was interpreted to be a secondary effect (151). Ultrastructure analysis revealed a reduction in the proportion of envelope relative to other chloroplast compartments in *dek5* (151), but a similar analysis was not done using *tic236* plants. More recently, a chloroplast outer

membrane protein CRL (crumpled leaf) was identified as a functional partner of Tic236 in the import of the plastid division machinery (27). Plastid division defects in *crl* and *tic236* mutants suggested that the CRL-Tic236 module is essential for plastid division (27).

2.2.4 Translocation at the inner envelope membrane

The discovery of a 1 MDa TIC complex has led to reconsideration of previously proposed models for the composition and operation of the TIC machinery (44; 53) (**Figure 2**). The Arabidopsis 1 MDa complex comprises Tic214 (encoded by the chloroplast gene *ycf1*), Tic100, Tic56, Tic21, Tic20 and Tic12. It is expected that the translocon channel is formed primarily by Tic20, which has four membrane-spanning α -helical domains (64). Three copies of Tic20 could theoretically exist within the 1 MDa complex (53), and so a pore size of up to 30-35 Å has been proposed (31). Tic12 is proposed to be part of the TIC core together with Tic20, and may be attached on the inside wall of the channel (153). Tic21 has a similar structure to Tic20 and may function in a complementary way (134). Recent genetic analysis provided *in vivo* support for the role of Tic100 in chloroplast protein import (84), and suggested that protein import is interlinked with intracellular communication via chloroplast retrograde signalling (84; 85). In fact, GUN1 (genomes uncoupled 1), a key player in several retrograde signalling pathways, enhances chloroplast protein import capacity through its interaction with cpHsc70-1 under environmental and genetic stresses and in early chloroplast biogenesis (48; 59; 132; 148).

Studies in *Chlamydomonas* identified a similar 1 MDa TIC complex with a proposed role in chloroplast protein import, and revealed a related proteostasis signalling response (chloroplast unfolded protein response, cpUPR) when the level of the 1 MDa complex was downregulated (108). Interestingly, several additional proteins of unknown function were associated with the TOC-TIC machinery in *Chlamydomonas*, suggesting that not all components of the chloroplast protein import machinery are yet identified, or that there are significant differences between land plants and algae in this regard (108). Protein translocation across the IEM in Rhodophyta may involve conserved mechanisms, and plastome-encoded Tic20 (12). Surprisingly, the 1 MDa TIC complex (along with the 2 MDa Ycf2-FtsHi complex; see below) is absent in grasses, which has raised questions about its functional significance (23; 112).

Numerous earlier studies had identified Tic110, Tic40, Tic20 and Tic21 as important components of the TIC apparatus, although mechanistic details and a description of the overall assembly were lacking (18; 22; 24; 42; 109). It is clear that both Tic110 and Tic40 play an essential role in chloroplast biogenesis (63), but the functional relationship between them and the 1 MDa complex is presently unclear (112). It is conceivable that the former are recruited to the latter or other import complexes during later stages of import, to co-ordinate chaperone functions or downstream targeting steps (14; 17; 37), and/or are required for the import of only some pre-proteins (66). Future studies are required to clarify the contributions and roles of the different TIC and chaperone systems.

2.2.5 Translocation motors

Significant energy is expended during protein translocation through the TIC complex into the chloroplast stroma (125). Several chaperones, including cpHsp70, Hsp90C and Hsp93, have been implicated in the provision of the motive force during protein import (112) (**Figure 2**). However, clarification is needed concerning the exact role of each chaperone. While cpHsp70 has been strongly linked to the role of main protein import motor (80; 128), Hsp90C was also found to be essential for protein translocation (38). A motor function was also proposed for Hsp93 (alternatively known as ClpC) (17), although data showing that it interacts with the ClpP proteolytic subunit at the

envelope imply that it may work mainly in a protein quality-control process at the point of import (30; 126).

More recently, a 2 MDa complex comprising Ycf2 (hypothetical chloroplast open reading frame 2) and several FtsHi (FtsH-inactive) ATPase subunits was identified in association with the 1 MDa TIC complex, as well importing preproteins, and it was proposed to function as a protein import motor (52) (Figure 2). The complex also contains and is stabilized by a moonlighting plastidic NAD-dependent malate dehydrogenase subunit (pdNAD-MDH), for which enzymatic activity is dispensable in this context (119). Considering the evidence supporting an unusually large maximal pore size for the TOC-TIC import channel (32), it is conceivable that such a powerful ATPase is recruited specifically to handle the import of recalcitrant or tightly-folded proteins (35). A *Chlamydomonas* orthologue of Ycf2, Orf2971, was found in association with several TIC, FtsHi and chaperone proteins, suggesting a role in chloroplast protein translocation and quality control (149). However, the role of the 2MDa complex in chloroplast protein import, and those of the stromal chaperone systems, have been strongly debated (69; 95).

2.3 Internal routing of chloroplast proteins

Chloroplasts are unusually complex organelles with several distinct suborganellar compartments: three different membranes (the two envelope membranes and the internal thylakoid membrane) and three discrete aqueous compartments (the intermembrane space of the envelope, the stroma and the thylakoid lumen). Thus, internal sorting of chloroplast proteins is necessarily complex (Table 2).

As noted earlier, most OEM proteins lack a cleavable TP, with targeting information instead residing within intrinsic hydrophobic transmembrane domains (56). In contrast, IEM proteins typically possess a TP and are imported via the TOC-TIC machinery. Two different targeting routes are reported: the stop-transfer and post-import pathways (141). In the post-import pathway, complete translocation into the stroma precedes membrane insertion in a separate event. This is similar to the conservative sorting pathway in mitochondria, so-called because it uses machinery of prokaryotic origin. In fact, components related to the bacterial Sec (secretory) apparatus have been identified in the plastid envelope, revealing that a second chloroplast Sec system (cpSec2) exists, in addition to the well-known cpSec1 system in the thylakoids (see below) (127). Further analysis showed that two IEM proteins (FtsH12 and Tic40) are translocated by the envelope cpSec2 apparatus (73; 74). In contrast, the stop-transfer pathway involves the lateral exit from the TIC translocon, which is mediated by client transmembrane regions, and may be important for aggregation-prone, hydrophobic proteins (141).

Structurally simple thylakoid membrane proteins may insert into thylakoid membranes via the so-called spontaneous insertion pathway, which does not seem to require energy input or the assistance of transport machinery (15; 121). Other thylakoid membrane proteins may follow the cpSRP (chloroplast signal recognition particle) pathway or the cpGET (chloroplast guided entry of tail-anchored protein) pathway. The cpSRP pathway mediates the targeting and insertion of LHCPs (light-harvesting chlorophyll-binding proteins), a highly-abundant group of polytopic membrane proteins. The cpSRP apparatus includes the broadly-conserved GTPase cpSRP54 but, unlike SRP systems in other contexts, no RNA. Other components are the plastid-specific chaperone cpSRP43, a stromal ankyrin protein LTD (LHCP translocation defect protein) that routes LHCP to the cpSRP pathway, the membrane receptor cpFtsY, and the YidC/Oxa1-related integrase Alb3 (albino3) (15; 45; 103; 121). In addition, components of this pathway and the cpSec1 pathway cooperate to enable the co-translational insertion of plastome-encoded proteins (111). Lastly, the cpGET pathway

delivers tail-anchored proteins (including components of the cpSec1 pathway; see below) to the thylakoid membranes, and involves the targeting factor ATPase Get3B (6). The receptor and the translocase/insertase of this pathway are currently unknown, although Alb3 and/or Alb4 could be involved due to their domain similarity to corresponding components in the cytosolic GET-targeting pathway (6).

Thylakoid lumen proteins follow the cpSec1 (chloroplast secretory translocase 1) or cpTat (chloroplast twin-arginine translocase) pathways, and possess bipartite targeting information: a standard TP precedes a luminal targeting peptide (LTP) that is similar to bacterial signal peptides (5; 15; 121). In both cases, the TP is removed upon emergence into the stroma, and the LTP is removed upon arrival in the lumen by the thylakoidal processing peptidase (91). The cpSec1 pathway involves the cpSecA1 ATPase motor and the cpSecY1-cpSecE1 translocon, and it accepts only unfolded proteins (5; 29); it is potentially facilitated by the chloroplast chaperonin Cpn60 (26; 58). By contrast, the cpTat pathway is powered by the thylakoidal proton gradient and may accommodate clients that acquire a folded structure in the stroma through cofactor binding or oligomerization. The cpTat apparatus comprises Tha4 (thylakoid assembly 4), Hcf106 (high chlorophyll fluorescence 106), and cpTatC, which are homologues of bacterial TatA, TatB and TatC, respectively (44; 98). In addition, a pair of ankyrin repeat proteins, STT1/2 (sorting of cpTat substrates to thylakoid membranes 1/2), form a heterocomplex that selectively targets cpTat substrates to the thylakoid translocon via a liquid-liquid phase separation mechanism (104). Substrates of the two pathways use similar but different LTPs; a twin-arginine motif in the N-terminal region of cpTat LTPs functions as the Sec-avoidance motif (29; 98). Differences in mechanism and pore architecture between the two pathways were revealed by measuring membrane conductance behaviour, suggesting that Sec translocation occurs through a barrel-stave-type proteinaceous pore, while Tat translocation likely occurs through a toroidal pore (10).

3. UBIQUITIN-DEPENDENT AND -INDEPENDENT PLASTID PROTEOLYSIS

As an endosymbiotically-derived organelle, the chloroplast contains ~20 proteases of prokaryotic origin, comprising >50 components, which act to maintain internal protein homeostasis (99). Activity of these proteases controls protein abundance or maturation, recycles cleaved targeting peptides, and mitigates photooxidation effects by the removal of damaged components (44).

The UPS is a preeminent system for the targeted removal of misfolded or unnecessary proteins in eukaryotes. Ubiquitination (or ubiquitylation) is a post-translational modification involving the addition of one or more copies of the 8.5 kDa ubiquitin protein to lysine (or other) residues of a target protein (90; 142). The addition of polyubiquitin chains targets the protein for degradation by the nucleocytoplasmic 26S proteasome (26SP) (142). Importantly, the cytosolic UPS also targets organelles, most prominently the ER through ER-associated protein degradation (ERAD) (81), but as has become increasingly clear recently, also the endosymbiotically-derived mitochondria and chloroplasts (77; 112).

Ubiquitination acts by marking its targets with ubiquitin tags through a cascade of reactions involving three key enzymes: E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligating) enzymes (142). Iterative rounds of conjugation via ubiquitin lysine residues result in the formation of a polyubiquitin chain, which acts as a degradation signal. Upon degradation, the ubiquitin moieties are recycled through the action of deubiquitinating enzymes. The E3 ligases are necessarily numerous and diverse, given their role in specificity, with roughly 1400 E3 proteins in *Arabidopsis*, far outnumbering the ~40 E2 and two E1 enzymes (77). In plants, there are four classes of E3 ligase: homologous to the E6-AP carboxyl terminus (HECT), really interesting new gene (RING),

U-box, and cullin-RING ligase (CRL) (77). Each class has a different mechanism of action and subunit composition, with particular diversity in the substrate-interacting domain or component (142).

Interestingly, in organisms with red complex plastids derived through secondary endosymbiosis (which typically have four bounding membranes), ubiquitination can be uncoupled from proteasomal degradation to mediate protein targeting. In the SELMA (symbiont-derived ERAD-like machinery) system, ERAD/UPS components have been repurposed to mediate pre-protein translocation across the second outermost plastid membrane (33).

3.1 Internal plastid proteases of prokaryotic origin

A variety of proteases operate inside plastids, and several subunits of these systems are encoded by multigene families enabling the formation of hetero-oligomers. Plastid proteases delivering protein turnover that have received particular attention include Clp (caseinolytic protease), FtsH (filamentation temperature-sensitive H), Lon (long-filament phenotype), and Deg (degradation of periplasmic proteins) (140) (Table 3). The Clp proteases comprise separate chaperone and serine protease subunits, are stromal, and may fulfil general housekeeping functions (83). By contrast, FtsH proteins are zinc-metalloendopeptidases that possess both chaperone and proteolytic functions within a single polypeptide, and are frequently thylakoid-associated; they play roles in the biogenesis of thylakoid membranes, the photosystem II repair cycle, and retrograde signalling (50). Lon proteases are broadly related to FtsH but lack transmembrane domains (99); nonetheless, Lon4 is attached to the stromal side of the thylakoid membrane (99). The Deg family are serine proteases and are involved in the degradation of photodamaged photosynthetic proteins and in the biogenesis of photosystem II (120). Notably, Clp chaperones, FtsH, and Lon all belong to the ubiquitous AAA+ family of ATPases, whereas Deg is ATP-independent.

Protease-chaperone interactions play important roles in regulating protein turnover in chloroplasts. For example, specific J-proteins and Clp chaperones cooperate with Hsp70 to deliver substrates to the Clp protease for degradation (107). Furthermore, structural analysis of the chloroplast Clp complex in *Chlamydomonas* revealed that the chloroplast Cpn11/20/23 co-chaperonin, a co-factor of Cpn60, forms a cap that inhibits Clp proteolytic activity, coordinating protein folding and degradation (144). It was also found recently that Cpn60 interacts with FtsH11, and thus potentially has a role in protein degradation (2).

Intriguingly, a recent study showed that foreign, misfolded proteins in the cytosol can be recognized by the chloroplast import machinery and taken up for degradation, through the action of SPP, Clp and FtsH proteases (82). This suggests a chloroplast-mediated cellular proteostasis mechanism analogous to MAGIC (mitochondria as guardian in cytosol), in which aggregated proteins in the cytosol are imported into mitochondria for degradation (113).

3.2 Ubiquitin-dependent degradation of chloroplast pre-proteins

Chloroplast-targeted pre-proteins can be marked for UPS-dependent degradation whilst still present in the cytosol (Figure 3, Table 4). In addition to preventing the accumulation of toxic cytosolic aggregates, this can help to shape the chloroplast proteome. Degradation of chloroplast pre-proteins by the UPS was first shown for the ClpP and FtsH precursors, and found to be mediated by the CHIP (C-terminus of Hsc70-interacting protein) E3 ligase (123; 124). A subsequent study revealed that in the Arabidopsis Toc159 mutant, *plastid protein import2* (*ppi2*), the cytosolic accumulation of pre-proteins was accompanied by elevated expression of the cytosolic chaperone Hsc70-4. Moreover, the chaperone interacted with the TPs of pre-proteins, recruiting them to CHIP for ubiquitination and degradation by the 26SP (68). Later, an Arabidopsis homologue of BAG proteins,

BAG1, was identified as a cofactor in such Hsc70-mediated pre-protein degradation (67). Another cytosolic E3 ligase, SAP5 (stress-associated protein5), identified in wheat, interacts with Hsp90C precursors to trigger their degradation (152). The regulatory significance of the UPS-dependent degradation of pre-proteins was highlighted by the finding that mild proteasome impairment, and the consequently enlarged pool of cytosolic precursors, leads to increased levels of functional photosynthetic complexes and photosynthetic performance (34). Interestingly, chloroplast pre-protein degradation through an endosome-to-vacuole pathway was recently uncovered in rice. Upon heat stress, the plasma membrane localized ubiquitin E3 ligase TT3.1 (thermo-tolerance 3.1) is relocated to endosomes where it ubiquitinates the chloroplast TT3.2 pre-protein; the latter is delivered to endosomes for vacuolar degradation, protecting chloroplasts from heat stress (150).

Regulation by the UPS of cytosolic events controlling chloroplast biogenesis also occurs during early plant development (Figure 3, Table 4). Seed germination is inhibited by DELLA regulatory proteins through the UPS-dependent regulation of developmental signals (72). The hormone gibberellic acid (GA) inhibits DELLA factor accumulation to enable germination and, in turn, chloroplast biogenesis. Under low GA conditions, DELLA proteins bind to cytosolic Toc159 prior to its assembly into TOC complexes, initiating its degradation by the 26SP (122). The UPS-dependent destruction of pre-proteins also occurs under such low GA conditions (122).

The UPS also affects chloroplast development indirectly through nuclear activities (88; 114; 133; 135), for example via the golden2-like (Glk) transcription factors Glk1 and Glk2, in Arabidopsis and tomato respectively (Figure 3, Table 4). Such golden2/Glk factors promote the expression of nuclear photosynthetic pre-protein genes, thereby promoting chloroplast biogenesis (135). The expression Glk1 is coordinated with plastid protein import by GUN1-dependent retrograde signals that report on the developmental state of the organelle (48). Interestingly, Glk1 is also regulated posttranslationally by the UPS in response to an as yet unknown, GUN1-independent plastid signal; this may contribute to the regulation of chloroplast biogenesis in response to environmental or developmental signals (135).

3.3 Chloroplast-associated protein degradation (CHLORAD)

Initial evidence for direct UPS action on chloroplasts came from the discovery and analysis of SP1 (suppressor of *ppi1* locus1), a RING-type ubiquitin E3 ligase located in the chloroplast outer envelope membrane (76). The SP1 protein directly interacts with all TOC proteins, mediating their ubiquitination and degradation (Figure 3, Table 4). Consequently, there is an increase in TOC protein abundance when SP1 is lost, which in the chlorotic *ppi1* (Toc33 mutant) background causes enhanced greening (i.e., suppression of *ppi1*) – a phenotype that enabled the identification of SP1, and which is mirrored by UPS inhibition (76). Such degradation of TOC complexes enables vital control of plastid protein import and, in turn, the organelle's proteome and functions; this occurs in response to developmental cues and environmental stresses, not only in Arabidopsis but also in crops (76; 78; 79).

To be degraded by the cytosolic 26SP, polyubiquitinated TOC proteins must first overcome the physical and energetic barriers to their removal from the OEM. In a subsequent study, SP1 was shown to operate within a multi-component pathway named CHLORAD (chlroplast-associated protein degradation) (75) (Figure 3, Table 4). In this, a retrotranslocation system comprising SP2 (an Omp85-type β -barrel channel, like Toc75) and CDC48 (a AAA+ ATPase motor) delivers ubiquitinated TOC proteins to the cytosol for proteasomal degradation. Evidence that UBX-domain-containing CDC48 adaptor protein exists in chloroplasts has been presented, but whether it acts in CHLORAD is unknown (25).

Recently, defects in CDC48 and its cofactors, UFD1 (ubiquitin fusion degradation 1) and NPL4 (nuclear protein localization 4), were found to cause the accumulation of ubiquitinated chloroplast proteins in Arabidopsis (71), pointing to a more direct role for the UPS in governing the chloroplast proteome. Indeed, comprehensive ubiquitinomic analyses of purified chloroplasts uncovered how photosynthetic apparatus components, and many other proteins in the chloroplast interior, are ubiquitinated (130). Further analysis demonstrated how these internal substrates are degraded by the 26S proteasome in the cytosol following extraction from the organelle by the CHLORAD machinery (i.e., SP2 and CDC48) (130) (Figure 3, Table 4). Accordingly, disruption of CHLORAD caused imbalances in photosynthetic activity as well as other chloroplast functions. Thus, the reach of the UPS extends to the interior of semi-autonomous chloroplasts where it acts to regulate one of the most fundamental processes of life (130). Significantly, a number of the newly identified ubiquitin targets are encoded by the plastome (71; 130), indicating that the modification must happen internally.

Deubiquitinases (DUBs) trim or remove ubiquitin chains, providing a vital extra layer of regulation to the UPS. However, what role(s) DUBs might play in CHLORAD is currently unknown. Interestingly, a recent study in *Chlamydomonas* identified a mating-type-linked DUB, Otu2p (otubain protein 2), that somehow prevents the UPS-mediated degradation of TOC proteins in *plus* gametes but not in *minus* gametes lacking Otu2p. As a result, *plus*-specific Otu2p establishes uniparental chloroplast DNA inheritance (46). Whether this is a CHLORAD-related function remains to be determined.

The stability of TOC proteins is not only determined by ubiquitin-dependent processes (i.e., CHLORAD), but also by the SUMO (small ubiquitin-like modifier) system. Modification with SUMO regulates TOC proteins in two distinct contexts. In the first, it acts prior to TOC assembly in the envelope, by modifying the aforementioned DELLA-mediated degradation of Toc159 precursors during seed germination (1; 122). In the second, it acts to promote the degradation of operational TOC components in a process that may be directly connected to CHLORAD (145).

4. CONCLUDING REMARKS

The chloroplast is a dynamic and complex organelle that functions as a central metabolic hub in the plant cell. Because of this, chloroplast proteostasis – taking in both protein translocation and proteolysis – is a topic of vital importance, and one that warrants thorough investigation. Doing so will not only deliver unique advances in our understanding of the fundamental principles of cell biology, but it will also help us to meet global agricultural demands.

As we have seen, most chloroplast proteins are delivered via the canonical TOC-TIC route, although an assortment of non-canonical import pathways also operate in parallel. Concerted efforts over several decades have now identified many components of the TOC-TIC apparatus, and of associated chaperone and motor systems, even though several major questions remain unresolved. Reflecting the fact that chloroplasts are structurally complex organelles comprising several distinct sub-compartments, multiple internal sorting pathways have been identified that operate downstream of the initial import step, and these play a vital role. Equally, various events that precede protein import have been uncovered, and shown to be important. One such event is the proteolytic regulation of cytosolic pre-proteins by the UPS. In fact, proteolytic regulation in chloroplasts is remarkably complex, as organelle-resident proteins are subject to control by both internal proteases of endosymbiotic origin and the cytosolic UPS via the CHLORAD pathway.

In spite of the considerable strides we have taken in recent years, many knowledge gaps exist concerning these aspects of chloroplast proteostasis. For example, our understanding of the

mechanisms and importance of the non-canonical pathways, and of possible co-translational chloroplast protein targeting, is still in its infancy. With regard to the TOC-TIC system, how pre-proteins are delivered across the IEM, and exactly how such translocation is powered by ATP hydrolysis, are subjects of ongoing debate. Moreover, little is known about the molecular structure of the TOC-TIC machinery. Structural analysis of purified or reconstituted protein import translocons will be required to elucidate how this supermolecular assembly operates mechanistically. Further studies are also needed to better understand the interactions between chloroplast protein import, quality control processes in the cytosol and organelle, and retrograde signalling. Recent developments of particular interest relate to the participation of the nucleocytosolic UPS in chloroplast proteostasis, and in this area much remains to be discovered. For example, a complete inventory of molecular components and knowledge of the underlying molecular mechanisms will be needed to fully understand CHLORAD. It will also be of great interest to delineate how the UPS and autophagy work together to shape the chloroplast proteome.

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DISCLOSURE STATEMENT

The application of CHLORAD as a technology for crop improvement is covered by a patent application (no. WO2019/171091 A).

RIGHTS RETENTION

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FIGURE LEGENDS

Figure 1

Overview of the different protein import and routing pathways in chloroplasts.

Most nucleus-encoded proteins enter the organelle following translation (by cytosolic 80S ribosomes) through the TOC-TIC (translocon at the outer/inner envelope membrane of chloroplasts) machinery (A); upon arrival, the transit peptide is removed by the stromal processing peptidase (SPP). That said, evidence is emerging that some proteins at least are imported co-translationally (B). Following import, intraorganellar sorting to the inner envelope membrane (IEM) or thylakoids may occur (C). Targeting to the IEM occurs via two pathways: the stop-transfer (i) and post-import (ii) pathways. Targeting to the thylakoids occurs via multiple pathways: two leading to the thylakoid lumen (cpSec1 and cpTat), and three leading to the membrane itself (cpSRP, cpGET and spontaneous). Lumenal targeting peptides are removed by the thylakoidal processing peptidase (TPP). In addition, plastome-encoded proteins are co-translationally targeted to the thylakoids by components of the cpSec1 and cpSRP pathways. Unlike IEM proteins, outer envelope membrane (OEM) proteins typically do not have transit peptides, and instead are targeted to the membrane by intrinsic targeting information held within transmembrane regions (A). A minority of internal proteins are imported by non-canonical pathway(s) that also employ intrinsic targeting information (D). Another unusual pathway involves co-translational translocation into the ER (endoplasmic reticulum) and passage through the Golgi (where glycosylation may occur) (E). Where known, requirements and key mediators of each pathway are indicated.

Figure 2

The TOC-TIC machinery responsible for canonical, transit peptide-mediated protein import.

Most nucleus-encoded chloroplast proteins are imported via the TOC and TIC multiprotein translocons located in the outer and inner envelope membranes (OEM and IEM, respectively). The core TOC complex comprises two transit peptide receptors, Toc159 and Toc34/33, and channel-forming β -barrel protein, Toc75. The receptors possess cytosolic GTPase domains, accounting for a GTP requirement during the early stages of import; Toc159 additionally possesses an N-terminal acidic (A) domain that may contribute to transit peptide recognition specificity. Different models exist for the TIC translocon. In one model, a 1 MDa TIC complex incorporating plastome-encoded Tic214 (or Ycf1) and four nucleus-encoded subunits (Tic100, Tic56, Tic21, Tic20 and Tic12) has a central role. In other models, Tic110 and Tic40 play important roles, but it is generally agreed that Tic20 has a central, channel-forming function. The Tic110 and Tic40 proteins are proposed to recruit and regulate a variety of stromal chaperones (cpHsp70, Hsp90C and Hsp93/ClpC), which drive protein import and/or assist in protein folding at the expense of ATP hydrolysis. Alternatively, a 2 MDa motor complex, comprising plastome-encoded Ycf2, five FtsH-like ATPase subunits, and a stabilizing NAD-dependent malate dehydrogenase subunit (pdNAD-MDH; indicated with an asterisk), is proposed to act as the import motor. The latter may be required to deliver the import of recalcitrant or tightly-folded pre-proteins.

Figure 3

Overview of the different steps at which the ubiquitin-proteasome system regulates chloroplast proteostasis.

The ubiquitin-proteasome system (UPS) regulates the assembly and composition of the chloroplast proteome in a variety of different ways. Firstly, in the nucleus, transcription factors such as Glk1/2

(which regulates the expression of photosynthetic pre-protein-encoding genes) may be degraded by the UPS in response to retrograde signals that report on the developmental or metabolic status of the organelle. Secondly, unimported pre-proteins in the cytosol may be processed by the UPS, to prevent their accumulation and the formation of cytotoxic aggregates; this is mediated by the chaperone Hsc70-4 and the E3 ligase CHIP. Thirdly, unassembled Toc159 in the cytosol may be degraded by the UPS, following the binding of DELLA factors, to repress precocious chloroplast biogenesis before germination under low gibberellic acid (GA) conditions. Lastly, chloroplast-resident proteins may be targeted by the CHLORAD (chloroplast-associated protein degradation) system during particular phases of development or in response to environmental stress. The TOC apparatus is a well-established target of CHLORAD, with ubiquitination being mediated by the E3 ligase SP1. Ubiquitinated TOC proteins are retrotranslocated from the membrane via the channel protein SP2, with motive force being provided by the cytosolic AAA+ ATPase CDC48. By targeting the TOC apparatus, CHLORAD is able to regulate chloroplast protein import and, therefore, the composition of the organellar proteome. In addition, recent evidence has shown that CHLORAD also acts to change the chloroplast proteome directly, by targeting a broad range of other proteins (substrates) in the OEM and in internal compartments of the organelle. In all cases, final degradation is mediated by the nucleocytosolic 26S proteasome (26SP).

Table 1 Different pathways targeting proteins to chloroplasts.

Pathway	Targeting signal(s); type	Comments, features	Example clients ¹
TOC-TIC pathway (44)	Transit peptide (TP); cleavable	Client proteins are synthesized as precursors (i.e., pre-proteins) and are processed by SPP upon import; key components are the TOC and TIC translocons	Employed by the majority of plastid proteins; RbcS is a commonly used model client
Simple OEM pathway (55)	Transmembrane domain; intrinsic	Targeting information resides within simple α -helical transmembrane domains; key targeting factors are AKR2 and Hsp17.8; Toc75 may be involved	Toc33/34, OEP7/14, OEP9, SP1
β -Barrel OEM pathway ² (22; 60)	Bipartite TP and/or β -signal; cleavable and/or intrinsic	Some β -barrel proteins (e.g., Toc75) possess cleavable targeting information, including a TP for TOC-TIC engagement; others do not	Toc75, OEP80, OEP21, OEP24, OEP37, OEP40
Co-translational pathway (129; 139)	Unknown (TP?)	A domain of the chloroplast envelope is bound by translating ribosomes	LHCP, RbcS
Non-canonical pathway (7; 92; 93)	Intrinsic targeting signal; intrinsic	The N-terminus of client proteins may not be required for targeting; translocation may be TOC-TIC-independent	ceQORH, IEP32/Tic32, GOX1, PDI1, GSTF2, Tic100
Endomembrane pathway (57; 97; 143)	Signal peptide for ER translocation, unknown for downstream steps; cleavable, unknown	This pathway enables glycosylation of client proteins; downstream targeting steps leading to the chloroplast are unknown	CAH1, NPP1, Amyl-1

¹ Abbreviations: ceQORH (chloroplast envelope quinone oxidoreductase homologue), IEP32 (inner envelope protein, 32 kDa), GOX1 (glycolate oxidase 1), PDI1 (protein disulphide isomerase 1), GSTF2 (glutathione S-transferase phi 2), CAH1 (carbonic anhydrase 1), NPP1 (nucleotide pyrophosphatase/phosphodiesterase 1), Amyl-1 (alpha-amylase isoform I-1).

² Unlike the other pathways in this table, this pathway is not explicitly shown in Figure 1.

Table 2 Different internal routing pathways inside chloroplasts.

Pathway	Destination sub-compartment	Comments, features	Example clients ¹
Stop-transfer (141)	IEM	Involves lateral exit from the TIC translocon; client transmembrane domain functions as a topology determinant	APG1
Post-import, cpSec2 (73; 74)	IEM	Involves complete translocation into the stroma prior to membrane insertion in a separate event	Tic40, FtsH12
cpSec1 (5; 29)	Thylakoid lumen	Client proteins possess bipartite targeting information; accepts only unfolded client proteins; is powered by ATP hydrolysis at cpSecA1	OE33/PsbO, Plsp1
cpTat (44; 98; 104)	Thylakoid lumen	Client proteins possess bipartite targeting information, and the LTP contains a twin-arginine motif; may tolerate client proteins in a folded structure; is powered by the thylakoidal proton gradient	OE17, OE23, PsaN, PsbT
cpSRP ² (15; 121)	Thylakoid membrane	cpSRP lacks the associated RNA typical of other systems; cpSRP43 is a plastid-specific chaperone	LHCPs
cpGET (6)	Thylakoid membrane	Function of Get3B depends on its ability to hydrolyse ATP	cpSecE1
Spontaneous (15; 121)	Thylakoid membrane	Does not require energy input or transport machinery	Structurally simple thylakoid membrane proteins (e.g., PsaK, PsaG, PsbW, PsbY)
Co-translational insertion of the plastome-encoded proteins (111)	Thylakoid membrane	Mainly mediated by cpSRP54; a parallel cpSecA pathway exists (for integration of cytochrome <i>f</i>)	Plastome-encoded thylakoid membrane proteins (e.g., PsbA, PetB)

¹ Abbreviations: APG1 (albino or pale green mutant 1), OE17, OE23 (17 and 23 kDa subunits of the oxygen-evolving complex), Plsp1 (plastidic type I signal peptidase 1).

² Plastid-specific cpSRP43 does not interact with cpSRP54 in *C. reinhardtii* during LHCP targeting, suggesting that alternative strategies exist in algae.

Table 3 Major internal plastid proteases of prokaryotic origin.

Protease	Localization	Components in Arabidopsis	Type	Comments
Clp (83)	Mostly stroma, small fraction at the envelope	Chaperone subcomplex: ClpC1, C2, D Adaptors: ClpS1, F Proteolytic core: ClpP1, P3, P4, P5, P6; ClpR1, R2, R3, R4 Auxiliary: ClpT1, T2	ATP-dependent, hetero-oligomeric complex	ClpP is the serine-type proteolytic subunit. ClpR is not catalytically active. Clp degrades a wide range of substrates. ClpP1 is encoded by the plastome.
FtsH (50)	Thylakoid, envelope	Thylakoid: FtsH1, 2, 5, 8 Envelope: FtsH7, 9, 11, 12 Unknown: FtsH6	ATP-dependent, zinc-metalloendopeptidase, hexameric heterocomplex, membrane-integral	Thylakoidal FtsH functions in photodamaged photosystem II repair, unassembled protein degradation, and thylakoid formation. FtsH11 contributes to heat tolerance. FtsH12 (together with several proteolytically-inactive FtsHi subunits) is a component of the 2 MDa complex at the IEM. For FtsH6, location and function remain unclear.
Deg (120)	Thylakoid, stroma	Thylakoid lumen: Deg1, 5, 8 Thylakoid membrane, stromal side: Deg2 Stroma: Deg7	ATP-independent, serine-type endopeptidase, either homomeric (many) or heterohexameric complex (Deg5, Deg8)	Function in degradation of photodamaged D1 proteins, and biogenesis of photosystem II.
Lon (99)	Stromal side of the thylakoid membranes (for Lon4)	Lon1, 4	ATP-dependent, serine-lysine protease, hexameric or heptameric complex	Lon1 and Lon4 display dual localization to both chloroplasts and mitochondria.

Table 4 UPS-dependent pathways affecting chloroplast (pre-)proteins.

Pathway	Localization	Major components	Target(s)	Function(s)
Pre-protein degradation (67; 68; 152)	Cytosol	CHIP, Hsc70-4, BAG1, SAP5	Various pre-proteins, including those of ClpP4, FtsH1 and Hsp90C	Removing unimported pre-proteins to prevent toxic aggregation or remodel the plastid proteome
DELLA-Toc159 (122)	Cytosol	DELLAs	Toc159	Blocking precocious chloroplast biogenesis prior to the onset of germination
CHLORAD (75; 76; 130)	Chloroplast and cytosol	SP1, SP2, CDC48	TOC apparatus, as well as a broad range of internal proteins including PrfB3, PsaA and PsbC	Remodelling the plastid's proteome and functions during development transitions and in response to environmental stress
Transcription factor regulation (48; 135)	Nucleus	Unknown	Transcription factors including Glk1/2	Regulating photosynthetic gene expression in response to plastid retrograde and other signals





