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REVIEW

# Chloroplast protein import machinery and quality control

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Most chloroplast proteins are nucleus-encoded, translated on cytoplasmic ribosomes as precursor proteins, and imported into chloroplasts through TOC and TIC, the translocons of the outer and inner chloroplast envelope membranes. While the composition of the TOC complex is well established, there is still some controversy about the importance of a recently identified TIC complex consisting of Tic20, Tic214, Tic100, and Tic56. TOC and TIC form a supercomplex with a protein channel at the junction of the outer and inner envelope membranes through which preproteins are pulled into the stroma by the ATP-powered Ycf2 complex consisting of several FtsH-like ATPases and/or by chloroplast Hsp proteins. Several components of the TOC/TIC system are moonlighting proteins with additional roles in chloroplast gene expression and metabolism. Chaperones and co-chaperones, associated with TOC and TIC on the cytoplasmic and stromal side of the chloroplast envelope, participate in the unfolding and folding of the precursor proteins and act together with the ubiquitin–proteasome system in protein quality control. Chloroplast protein import is also intimately linked with retrograde signaling, revealing altogether an unsuspected complexity in the regulation of this process.

## Introduction

It is well accepted that chloroplasts arose more than a billion years ago through endosymbiosis in which a cyanobacterial ancestor was engulfed by an ancient eukaryotic cell and over time gradually became fully integrated into the host cell and its metabolism. In particular, a massive gene transfer occurred from the endosymbiont to the host nucleus. These events led finally to the formation of a functional chloroplast with a genome containing close to 100 protein genes, a few rRNA genes, and a complete set of tRNA genes. Concomitantly with this gene transfer, the proteins encoded by the cyanobacterial genes transferred to the nucleus had to be relocated back into the organelle to maintain its functions. This was achieved through the development of a chloroplast protein import machinery in the chloroplast envelope through which the nucleus-encoded organellar proteins translated on cytoplasmic ribosomes as precursor proteins could be specifically transported into the chloroplast. Most chloroplast proteins, ca. 2000 to 3000 proteins, are routed to the chloroplast in this way. The precursor proteins in the cytosol can be co- and post-translationally modified, and they interact with chaperones, co-chaperones, 14-3-3 protein, and/or E3 ligases before they are transported into chloroplasts through the TOC/TIC translocon. Alternatively, they may be degraded by the cytosolic proteasome complex in the case of disturbances of protein import and chloroplast

**Abbreviations**
AAA family, ATPases associated with various cellular activities; CHLORAD, chloroplast-associated protein degradation; cpUPR, chloroplast-unfolded protein response; Cytb6f, cytochrome b6f complex; NDH, NAD dehydrogenase; PhANG, photosynthesis-associated nuclear gene; PSI, photosystem I; TIC, translocon of inner chloroplast envelope membrane; TOC, translocon of outer chloroplast envelope membrane; UPS, ubiquitin–proteasome system.



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proteostasis. Upon import into chloroplasts, the precursor proteins are cleaved by the stromal processing peptidase before being folded and assembled in the stroma or guided further to the thylakoids or to the inner envelope membrane (Fig. 1). The chloroplast protein import machinery and its regulation have been discussed in several recent reviews [1–5]. Here, new developments in this field are presented and discussed.

## TOC-TIC complex

The chloroplast import machinery consists of the TOC (translocon of outer chloroplast envelope membrane) and TIC (translocon of inner chloroplast envelope membrane) multimeric complexes. The composition of the TOC complex has been studied in great depth. It consists of the preprotein receptors Toc159 and Toc33 with cytosol-exposed GTPase domains that bind to the transit peptides of the precursor proteins. These receptors have different isoforms in *Arabidopsis* named Toc132/-120/-90 and Toc34, respectively, with different client specificity. The third component of the TOC complex is Toc75, which forms the protein-conducting channel [1].

Earlier studies concluded that several proteins including Tic20, Tic 22, Tic21, Tic 40, and Tic110 form the inner membrane protein import complex (for review see [4]). Tic110 was originally proposed to bind precursor proteins during inner membrane translocation and to act as a scaffold for the recruitment of

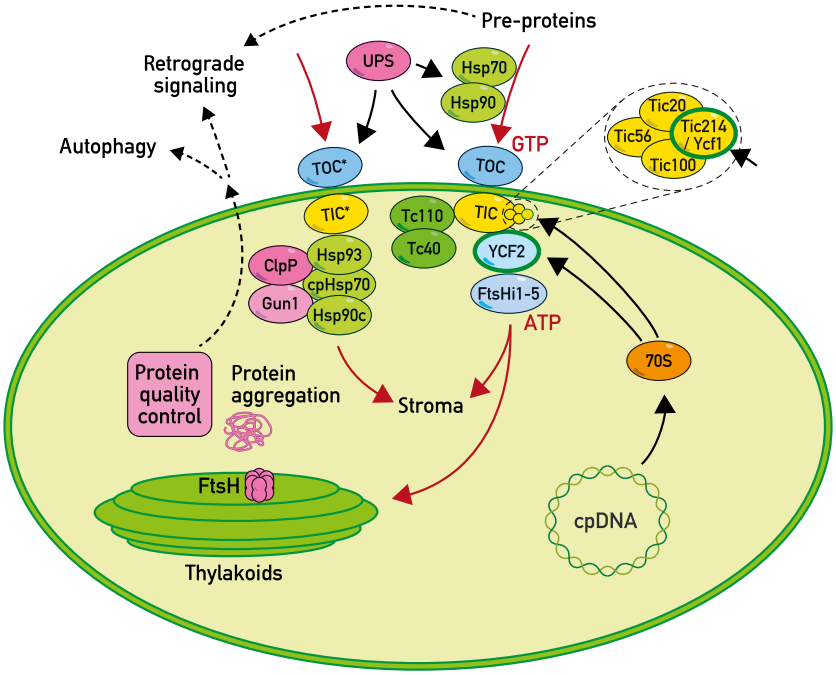


**Fig. 1.** Protein import into chloroplasts and its regulation. Nucleus-encoded chloroplast proteins are synthesized as precursor proteins in the cytoplasm and imported into the chloroplast through the TOC (consisting of GTPase receptors Toc 150 and Toc33 and the channel forming Toc75) and TIC translocons on the outer and inner envelope membrane, respectively. They are unfolded by cytosolic chaperones (Hsp70, Hsp90) and pulled through the TOC and TIC translocon channels as unfolded proteins by the ATP-powered chloroplast Hsp90 and Hsp70, and Ycf2 complex that are involved in protein refolding as the precursor proteins emerge into the stromal space. The core subunits of the 1 MDa TIC and Ycf2 complex are indicated. The Tic214 and Ycf2 subunits of these complexes (indicated with green frames) are encoded by chloroplast genes, translated on chloroplast 70S ribosomes, and directed to the chloroplast envelope. A second still largely unknown protein transport system must exist to account for all the experimental data (marked with *). The TIC* complex may consist of the Tic20-IV homolog associated with unknown partner proteins. Hsp93, cpHsp70, and Hsp90C could act as import motors for this complex. The proteins are then routed to the thylakoid membranes or the stroma. The role of Tic110 and Tic40 is not clear, and they may act downstream of TOC and TIC and participate in protein-folding. Under stress conditions (high light, perturbed chloroplast protein synthesis), aggregation of plastid proteins occurs. Protein aggregation in the chloroplast leads to the unfolded protein response (UPR), which is part of the chloroplast protein quality system [18]. These aggregates are degraded by chloroplast proteases (ClpP, FtsH) and can elicit via retrograde signaling to the nucleus increased expression of chloroplast chaperones, proteases, and UPS, and under extreme conditions autophagy. Accumulation of precursor proteins in the cytosol elicits upregulation of UPS. Gun1, a key factor in retrograde signaling, interacts with Hsp93/ClpC. Protein routes are indicated in red.

other stromal translocon components and of stromal chaperones to import sites [6–8] and later suggested to form a protein-conducting channel [9,10], but its role in the chloroplast translocon has been a matter of debate [11,12]. Recent structural studies indicate that it is most likely a scaffolding component important for protein–protein interactions to recruit other translocon components and chaperones in the stroma [13]. Moreover, this early TIC model has been recently revised with the identification of a 1 MDa TIC complex consisting of Tic20, Tic214, Tic100, and Tic 56 in *Arabidopsis* [14]. Surprisingly, Tic214, designated earlier as Ycf1, is encoded by the chloroplast genome of dicots and green algae, but absent in grasses. Although the presence of a chloroplast-encoded subunit in the chloroplast protein import machinery has raised some controversy [15–17], recent studies with *Chlamydomonas reinhardtii* fully confirm the existence of this TIC complex in green algae [18]. Indeed, co-immunoprecipitation experiments clearly revealed that Tic214 interacts with components of the TIC and TOC translocons in this alga [18]. In addition, studies on *in vitro* chloroplast protein import with *Chlamydomonas* established that Tic214 is part of the active translocon complex and that its association with protein translocation intermediates is stimulated by the presence of ATP in the import reaction [18]. In *Arabidopsis*, the 1 MDa TIC complex was found to be associated with translocating chloroplast precursor proteins and to display preprotein-dependent channel activity [14]. No significant interaction was detected between Tic 40 and Tic110 with this complex [14,18]. However, several additional proteins of unknown function were found to be associated with the *Chlamydomonas* TOC/TIC supercomplex that whad not been identified in similar studies with *Arabidopsis* [18]. The expression patterns of the genes of these uncharacterized proteins were found to be highly correlated with the expression patterns of known TOC and TIC components raising the possibility that these proteins represent novel factors involved in chloroplast protein import.

The absence of Tic 214 in grasses raises questions on the composition of the TIC complex in these species. Null mutants in the Tic translocon components Tic20, Tic100, and Tic 56 show severe seedling lethality but are still able to develop albino seedlings when grown on a sucrose-supplemented medium. In these seedlings, at least some plastid proteins involved in housekeeping functions are still imported and accumulate in plastids [14,19,20]. This residual protein import was correlated with increased expression of Tic20-IV, a minor isoform of Tic20 that is mostly expressed in roots [14,19] raising the possibility that it may be part of a second minor protein import system in the inner chloroplast envelope (Fig. 1).

It is surprising that although protein import is an essential process in chloroplast biogenesis and function, the sequences of the TIC components have been poorly conserved during evolution with sequence identities ranging between only 14% and 22% when comparing *Arabidopsis* with *Chlamydomonas* [18]. This is especially striking for Tic214. No homolog of Tic214 could be found in cyanobacteria, although chloroplast genomes are derived from an ancestral cyanobacterial genome and in some early lineages including Rhodophyta and glaucophytes [17]. The same holds for Tic 56 and Tic100 but not for Tic20 that is present in all plastid-containing lineages examined [17].

The TIC/TOC complex spans both the outer and inner envelope membrane and is remarkably stable in *Chlamydomonas* and *Arabidopsis* [18,21]. This complex must correspond to envelope contact sites between the two membranes, which have been observed by electron microscopy [22]. TOC-TIC supercomplexes have also been reported based on BNG after solubilization of chloroplasts in pea and *Arabidopsis* [23]. In *Arabidopsis*, Tic22 and Tic236 have been proposed to link the TOC and TIC translocons [24,25]. Although these two proteins are conserved in *Chlamydomonas*, none of them could be detected amongst the proteins co-immunoprecipitated with Tic20 and Tic214 and they were not associated with the 1 MDa TIC complex of *Arabidopsis* [14,18]. Thus, either Tic22 and Tic236 are not required to zip together the TIC and TOC complexes or they escaped detection amongst the immunoprecipitated proteins.

Other possible candidates for mediating membrane apposition are the peripheral components Tic56 and Tic100 with protrusions in the intermembrane space [12,14]. Indeed, Tic56 was shown to interact with TOC components independently of precursor translocation [21]. Moreover, the two MORN motifs in Tic100 may mediate the connection between the envelope membranes as MORN repeats are proposed to contribute to membrane binding by interacting with lipids [26]. In *Arabidopsis*, Tic 100 also contains two acidic segments that might be involved in transient interactions with the positively charged transit peptides of preproteins. Interestingly, these two acidic segments have been replaced with a highly phosphorylated region in the *Chlamydomonas* protein, possibly providing an alternative mechanism for recognizing the incoming preprotein [18].

Most of the genes encoding proteins associated with the TIC complex are highly co-regulated in both

*Chlamydomonas* and *Arabidopsis* and define a regulon of translocon genes [18]. Interestingly, this regulon includes additional genes that were not identified in the co-immunoprecipitation experiments. These genes may play an accessory role in chloroplast translocon assembly or regulation. Clearly, their possible role in protein import and quality control needs to be investigated. Remarkably, transcriptional co-regulation of translocon components is not only restricted to nuclear genes but also includes the chloroplast compartment, as the essential plastid-encoded translocon component Tic214 is co-expressed with nucleus-encoded Tic20. Other plastid genes coordinately expressed with *tic214* include *orf2971*/*ycf2* (see below) and the *rpo* genes encoding the subunits of the chloroplast RNA polymerase. The inclusion in this set of the *rpo* genes raises intriguing questions about possible links between protein import and chloroplast gene expression.

## ATP-driven motors for protein translocation

Energy is required to pull proteins through the TIC complex into the chloroplast stroma. Numerous studies led to the conclusion that chloroplast chaperones such as Hsp93 (also designated ClpC), cpHsp70, and Hsp90 are associated with the translocon and could function as protein import motors. The evidence was based on co-immunoprecipitations of these chaperones with translocon proteins and translocation intermediates [6,7,27–29] and on the observation that Hsp93 and cpHsp70 bind the transit peptide of precursor proteins [30,31]. Based on genetic analysis, it was proposed that two chloroplast import motors function in parallel, Hsp93 functionally associated with the cochaperone Tic40, and the cpHsp70 proteins in *Arabidopsis* [29]. The latter belongs to the 14-member Hsp70 protein family of which two are localized within chloroplasts and referred to as cpHsc70-1 and cpHsc70-2. Inactivation of either cpHsc70-1 or cpHsc70-2 led to reduced protein import efficiencies and the double mutant was lethal, suggesting that both cpHsc70s are important for protein import into chloroplasts, at least at specific stages of development (14- to 24-day-old plants) [29]. The biochemical analysis further showed that cpHsc70 is stably associated with the translocon and can be co-immunoprecipitated with precursors during import [29].

More recently, a 2MDa heteromeric complex associated with the TIC complex that functions as an ATP-driven protein import motor and that interacts with the imported preproteins was characterized in *Arabidopsis* [32]. This complex consists of a heterohexameric AAA-ATPase and includes the chloroplast-encoded Ycf2 protein, four nucleus-encoded FtsH-like proteins FtsHi1, FtsHi2, FtsHi4, FtsHi5, and FtsH12. All these components are essential and have retained the AAA-type ATPase domain (s), although only FtsH12 contains the zinc-binding site that is usually conserved among FtsH metalloproteases. However, this site can be removed without affecting protein import. These members of the Ycf2/FtsHi complex appear to have evolved from the chloroplast-encoded membrane-bound AAA protease of the ancestral endosymbiont [32]. In addition, the complex contains a NAD-malate dehydrogenase, which is required for stabilizing FtsH12, but its enzymatic activity is dispensable [33]. A homologous complex has been identified in *Chlamydomonas* containing the *orf2971* product, the homolog of Ycf2, and several FtsHi proteins that are closely associated with the TIC complex (J. Xing, J. Pan, H. Yi, K. Lv, Q. Gan, M. Wang, H. Ge, X. Huang, F. Huang, Y. Wang, J.D. Rochaix, W. Yang unpublished results). Similar to *Arabidopsis*, the algal complex contains several FtsH-like proteins with an AAA-type ATPase domain but lacks the zinc-binding motif critical for protease activity. These results suggest that the Ycf2-FtsHi complex acts as the import motor in *Chlamydomonas* and may also be involved in the folding of the imported proteins as they emerge into the stromal compartment. No association between Hsp93/ClpC, cpHsp70, or Hsp70 proteins and the Ycf2-FtsHi complex of *Arabidopsis* could be detected suggesting that these proteins act independently [32].

Thus, at least two chloroplast import motors have been identified based on robust experimental data. This suggests the existence of import motors associated with different types of TIC translocons in *Arabidopsis*. As mentioned above, one could be the 1 MDa TIC complex with Tic20-1 that is associated with the Ycf2/FtsHi complex as import motor, the other could be a complex containing Tic20-IV, and additionally still unidentified components with Hsp93, cpHsp70, and Hsp90C acting as import motors. Clearly, further studies are required to fully determine the composition and function of these complexes.

As Tic110 was not found in the *Arabidopsis* TIC 1 MDa complex [14] nor in the TIC-associated motor complex [32] and barely detected in the TIC/TOC complex of *Chlamydomonas* where its expression is only weakly co-regulated with other components of the translocon [18], it is possible that Tic110 is part of a different translocon complex, perhaps involved in pre- or post-translocation events and dynamically regulated in response to different cellular needs. However,

there is no question that Tic110 and Tic40 play key roles in chloroplast biogenesis as disruption of their genes leads to embryo lethality, and their precise role remains to be elucidated.

It is rather surprising that the chloroplast genes encoding Tic214 and Ycf2 are present in the plastid genomes of green algae and of most plants except for grasses. Moreover, Tic100 and Tic56 are also missing in grasses. Thus, alternative translocon complexes must exist in these species. In this respect, it should be noted that the genes of some translocon subunits have several paralogs as mentioned above for Tic20 of *Arabidopsis* [34]. Amongst these, Tic 20-I and Tic20-IV appear to have different substrate preferences [19]. Tic 20-1 would correspond to the major photosynthetic 1 MDa TIC complex responsible for the massive import of photosynthetic proteins, whereas Tic20-IV would be part of a minor alternative "non-photosynthetic" TIC complex presumably for housekeeping proteins [12,14]. It was proposed that during evolution, the major complex was lost in grasses and the minor complex became the predominant TIC translocon [2,17]. Determination of the composition of the *Arabidopsis* Tic20-IV containing non-photosynthetic complex and the grass-type TIC complex containing Tic20 is needed to explore this further. It is noteworthy that in *Arabidopsis tic56-1 tic20-IV and tic20-I tic20-IV*, double mutants are embryo-lethal [14], whereas the single mutants are not. These findings are compatible with the existence of distinct translocons with partially redundant functions. Another contentious issue is to what extent the loss of Tic56 and of the 1 MDa TIC complex in *Arabidopsis* affects protein import as contradictory results and interpretations have been presented [14,20,21,35]. Further studies will be necessary to fully understand the functional role of the different TIC complexes, but it is clear that the core composition of the translocon supercomplex is evolutionarily conserved from green algae to most higher plants.

## Impairment of chloroplast protein translocation triggers the chloroplast-unfolded protein response

A repressible chloroplast gene expression system of *Chlamydomonas* has been developed in which any chloroplast gene can be repressed by adding vitamins to the growth medium [36]. This genetic tool was applied to specifically repress the expression of *tic214* in *Chlamydomonas* [18]. The depletion of Tic214 drastically affected cell growth and survival confirming that Tic214 plays an essential role. As expected for a block in chloroplast protein import, unprocessed chloroplast precursor proteins containing cleavable transit peptides accumulated in the cytosol upon Tic214 depletion when the ubiquitin proteasome was inhibited, as it would otherwise fully degrade the precursor proteins and make them undetectable [37]. Rubisco was amongst the major proteins accumulating in the cytosol, forming structures resembling Rubisco-containing bodies (RCBs) in the cytoplasm [18].

Upon Tic214 depletion, accumulation of the majority of chloroplast proteins was decreased in *Chlamydomonas*. However, a group of nucleus-encoded chloroplast proteins increased transiently, both at the transcript and protein level. They include the chloroplast small heat shock proteins Hsp22E, Hsp22F, and Hsp22C, the membrane biogenesis factor Vipp2, the chaperones ClpB and Hsp93/ClpC, the chaperonin 20, a DegP-type protease, and several proteins with suggested roles in signaling. Hsp22E and Vipp2 are early markers of the chloroplast-unfolded protein response (cpUPR), a stress response pathway activated by impairment of chloroplast protein homeostasis [18,38,39]. Because most chloroplast proteins are nucleus-encoded and then imported into the organelle, the downregulation of the translocon imbalances the composition of multiprotein complexes, resulting in the accumulation of unassembled and unfolded proteins and possibly in protein aggregation. Similar to the UPR from other organelles, the transient induction of gene expression during the cpUPR likely reflects the cell's attempt to restore chloroplast functions that cease later because stress-relieving cpUPR target proteins can no longer be imported into the organelle upon inactivation of the translocon.

## Are some translocon components moonlighting proteins?

Several components of the TOC/TIC translocon and of the Ycf2 complex display protein import-independent functions. Thus, Tic214, in addition to its role as the core protein of the TIC complex, was also found to be the direct downstream target of PBR1 in *Arabidopsis*, a nucleus-encoded chloroplast RNA-binding protein that regulates the assembly of the NDH, PSI, and Cytb6f complexes [40]. PBR1 binds to the Tic214 mRNA to control its translation. Interestingly, relocation of the chloroplast Tic214 gene fused with a transit peptide sequence to the nucleus was sufficient to complement the defects in the biosynthesis of the photosynthetic complexes in plants lacking PBR1 [40]. Thus, AtTic214 plays an important role in this assembly process. In *Chlamydomonas*, the ortholog of Tic214 known as Orf1995 was found to bind nucleic

acids [41] and to be associated with the Ycf4 complex that plays a key role in PSI assembly [42]. These observations strengthen the view that this protein has additional roles in the assembly of photosynthetic complexes and perhaps nucleic acid metabolism besides its involvement in protein import.

Tic56, another component of the TIC complex, also appears to exert a dual role. Two mutants of Tic56 were characterized [20,21]. The first mutant, *tic56-1*, is a null mutant that does not accumulate the 1 MDa TIC complex; it is albino and cannot grow autotrophically. It is impaired in the processing of the chloroplast 23 S rRNA, which affects the assembly of functional ribosomes [20]. Thus, there might be a link between chloroplast protein import, processing of plastid RNA, and assembly of plastid ribosomes. However, it is not clear whether the deficiency in rRNA processing observed in *tic56-1* is directly due to the loss of Tic56 or to an indirect effect resulting from impaired protein import. The observation that other mutants with a similar albino phenotype, but carrying a different genetic lesion, are not affected in plastid RNA processing would argue against the latter possibility.

The *tic56-3* mutant produces low amounts of a truncated form of Tic56; it is pale-green but has no significant defect in photosynthetic activity. *In vitro* protein import assays revealed only a slight decrease in *tic56-3* compared with the wild type [14,21]. However, this is not surprising because the TDNA insertion in *tic56-3* is very near the end of the gene, and a truncated protein accumulates in significant amounts that may still be functional to some extent [14].

Another case of moonlighting protein is NAD-malate dehydrogenase, which is associated with the Ycf2 complex. It probably plays a structural role in this complex, although it is not clear how this is achieved at the molecular level. These examples show that during evolution, some proteins were recruited for improving chloroplast protein import while keeping their ancient function. Alternatively, some of the proteins such as Tic56 might have first been exclusive components of the protein import system before acquiring new roles in chloroplast protein or RNA metabolism.

## Chloroplast protein import and quality control, and retrograde signaling

Chloroplast protein import is impaired in several mutants of *Arabidopsis* affected in components of the TIC/TOC system such as *ppi1* deficient in Toc33, and *ppi2* deficient in Toc159 and tic56-1, in which the 1 MDa TIC complex no longer accumulates. As a result of this impairment, nonimported chloroplast precursor proteins accumulate in the cytosol causing folding stress in the cytosol and inducing a cytosolic protein quality control system. It comprises the ubiquitin–proteasome system (UPS) and the cytosolic Hsp70-Hsp90 chaperone complex that downregulates the expression of photosynthesis-associated nuclear genes (PhANG) [18,43,44]. This system may also target transcription factors such as Abi4 [45], Hy5 [46], and Glk1/2 [47], which are all involved in retrograde signaling.

Degradation of chloroplast precursor proteins by the ubiquitin–proteasome system (UPS) was first shown for the ClpP and FtsH protease precursors through their interaction with the CHIP E3 ligase under high light conditions [48,49]. These observations suggested a possible regulatory link between retrograde signaling by precursor proteins and their removal by UPS. Indeed, the accumulation of precursor proteins in the *ppi2* mutant was accompanied by increased expression of a cytosolic Hsp70 isoform that interacted with the transit peptides of precursor proteins and guided them to the CHIP E3 ligase for degradation by the proteasome [37]. A suppressor screen of the *ppi1* mutant revealed an E3 ubiquitin ligase-dubbed Sp1 [50]. This enzyme was shown to ubiquitinate components of the TOC complex and to target them for degradation. In this way, the protein import machinery can be remodeled for rapid adaptation to changing environmental conditions. Further evidence for a regulatory role of UPS in chloroplast protein import and biogenesis was provided by the genetic analysis of the *ppi2* mutant lacking Toc159. Double mutants lacking Toc159 and proteasome subunits accumulate increased amounts of functional photosynthetic complexes through a mechanism, which does not involve Sp1. Instead, it appears that the mild proteasomal impairment leads to an increase in cytosolic precursor proteins and thereby facilitates their import into plastids.

Gun1, a PPR-repeat containing protein associated with the chloroplast nucleoid and one of the central regulators of retrograde signaling, was recently shown to regulate chloroplast protein import through its interaction with the stroma-localized chaperone cpHsc70, one of the major molecular motors for pulling preproteins through the import channel [44]. In the presence of lincomycin, an inhibitor of plastid protein synthesis, or norflurazon that induces bleaching by blocking carotenoid synthesis, the *gun1* mutant displays a protein import defect, which causes the accumulation of precursor proteins and induces the cytosolic protein quality control system comprising the Hsp70 and Hsp90 proteins along with their

co-chaperones and proteasome subunits. In this case, however, PhANG expression is enhanced, in apparent contradiction with the observed downregulation of PhANG when chloroplast protein import was reduced in mutants deficient in protein import [43,44]. In this respect, it should be noticed that the Gun1 function during protein import is only apparent during early chloroplast development or when retrograde signaling is affected by mutations of specific components of the protein import system as in the *clpC1* mutant or by treating *gun1* seedlings with norflurazon and lincomycin. Indeed, Gun1 also interacts with the ClpC1 chaperone that associates the ClpP protease with the inner envelope membrane close to the protein import channels and is part of a plastid protein quality control system. In this way, defective or unwanted proteins can be degraded upon their arrival inside the chloroplast. Based on these results, it was proposed that Gun1, through its interaction with cpHsc70, enhances the chloroplast protein import capacity under environmental stress or during the early stages of chloroplast biogenesis [44].

Import of proteins through the TOC/TIC translocon varies considerably throughout the development and in response to environmental changes. The import process needs therefore to be tightly regulated, and in this regard, the UPS system plays a major role by acting at several levels including the selective degradation of transcription factors required for the coordinate expression of PhANG, the degradation of excess precursor proteins in the cytosol, and the remodeling of the TOC complex in response to developmental and environmental cues, a process termed chloroplast-associated protein degradation (CHLORAD).

Because cytosolic precursor proteins can trigger retrograde signaling, control of their accumulation by the UPS system is important for regulating chloroplast function. This appears to be mediated by specific interactions with Hsp70 or Hsp90 proteins and recognition by cytosolic E3 ligases such as the Plant U-box ubiquitin ligase Pub4 that regulates chlorophagy, a process that removes oxidatively damaged chloroplasts through ubiquitination of envelope proteins [50].

CHLORAD targets damaged components of the TOC complex [51]. In order to be degraded, these TOC subunits first need to be extracted from the outer chloroplast membrane before they are ubiquitinated by the chloroplast outer membrane ubiquitin ligase Sp1 and subsequently delivered to the 26S proteasome through the membrane channel protein SP2 and the $AAA^+$ ATPase Cdc48 [51]. As Sp1, Sp2 interacts directly with the TOC proteins, and it is thought to form a translocation channel. The driving force for extraction of Toc subunits through SP2 is provided by Cdc48, associated with Toc33 on the chloroplast envelope [52,53].

Another E3 ligase that targets precursor proteins in the cytosol for degradation via ubiquitination when protein import is defective is AtCHIP, which binds to the C-terminus of Hsc70 [37,48,49]. One of the targets of this E3 ligase is ClpP4, a nucleus-encoded core subunit of the chloroplast ClpP protease, which plays an important role in protein quality control. This remarkable finding reveals a direct role of the cytosolic ubiquitin-mediated degradation pathway in the regulation of the proteolytic activity inside chloroplasts.

Chloroplast biogenesis was shown to be repressed during early plant development by DELLA proteins that inhibit seed germination through UPS-mediated degradation [54,55]. The hormone gibberellic acid is known to prevent DELLA protein accumulation and to promote germination. Under low gibberellic acid conditions, DELLA proteins can interact with Toc159 before it is inserted into the TOC complex and initiate its degradation by the UPS.

Regulation of the stability of the TOC complex occurs not only by the ubiquitin-dependent chloroplast-associated protein degradation pathway but also by the small ubiquitin-like modifier (SUMO) system [56]. Mutants covering nearly the entire SUMO conjugation pathway could partially suppress the pale yellow phenotype of *ppi1* with respect to leaf chlorophyll accumulation, chloroplast development, and TOC protein abundance in a similar way as the E3 ubiquitin ligase mutant mentioned above. Overexpression of either SUMO1 or SUMO3 enhanced the severity of the *ppi1* phenotype. Moreover, the E2 SUMO-conjugating enzyme, SCE1, and other SUMO proteins were found to physically interact with TOC proteins based on bimolecular fluorescence complementation experiments and immunoprecipitation assays [56]. Taken together, these studies indicate that several TOC proteins are SUMOylated under some stress conditions and also during specific developmental stages [57].

Another aspect to be considered is that besides the interactions between UPS and the accumulated precursor proteins in the cytoplasm, the assembly of the photosynthetic complexes is compromised because the nucleus-encoded subunits cannot be delivered leading to the accumulation of unassembled chloroplast-encoded partner subunits. In this way, the capacity of the chloroplast proteolytic system represented mainly by the stromal ClpP, and thylakoid FtsH proteases may be exceeded. As a result, chloroplast protein homeostasis is perturbed accompanied by protein

aggregation, a situation which was also encountered when ClpP was depleted in *Chlamydomonas* [38]. In the latter case, this disturbance of proteostasis led to the chloroplast-unfolded protein response, which involves the repression of most nuclear genes with exception of several chaperones and stress proteins such as the small Hsp proteins. In fact, the set of upregulated proteins upon Tic214 depletion or ClpP depletion overlap to a large extent [18,38] suggesting that the corresponding signaling chains may converge at some point (Fig. 2). To identify the components of this signaling chain, a genetic screen chain was performed with *Chlamydomonas* [39]. The first characterized signaling mutant is deficient in Mars1, a cytosolic protein kinase. Loss of Mars1 prevents induction of the cpUPR and impairs the ability of the cells to cope with chloroplast stress such as protein aggregation and exposure to high light. Interestingly, the activation of the cpUPR through Mars1 mitigates photooxidative stress and delays photobleaching. Mars 1 is a large protein with no known motifs except for a Ser/Thr kinase domain at its C-terminus. Although no ortholog of Mars1 has yet been identified in land plants, the question of whether a functionally related protein kinase exists in these organisms remains open. A major challenge will be to identify the signals and components of the signaling chains depicted in Fig. 2 and to understand how they are coordinated with each other.

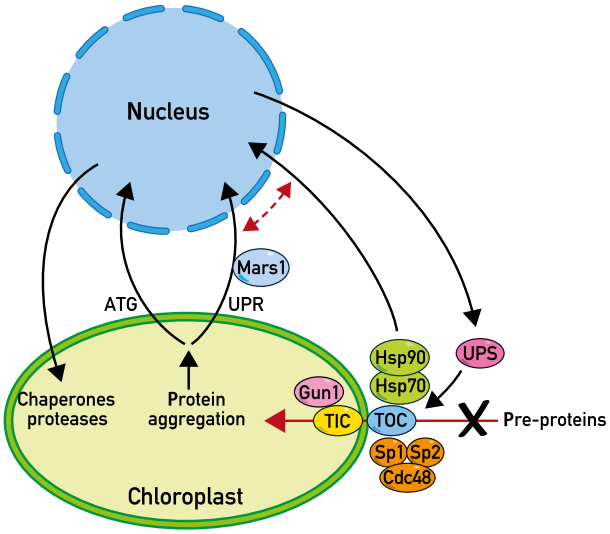


**Fig. 2.** Impairment of chloroplast protein import induces several signaling pathways. Impairment of chloroplast protein import leads to the accumulation of nucleus-encoded chloroplast precursor proteins in the cytosol that triggers a nuclear response involving UPS and the Hsp70 and Hsp90 chaperones. In the chloroplast, assembly of the photosynthetic complexes is compromised because the nucleus-encoded subunits cannot be delivered leading to the accumulation of chloroplast-encoded partner subunits that may exceed the capacity of the chloroplast proteolytic system represented mainly by ClpP and FtsH. As a result, protein aggregation occurs in the chloroplast that triggers the chloroplast unfolded protein response and chloroplast autophagy. These responses involve the induction of several chaperones and stress proteins through retrograde signaling. Except for the cytosolic Mars1 protein kinase, the other components of the UPR signaling chain are still unknown.

## Conclusions

The field of chloroplast protein import has greatly expanded in recent years. While there is a large agreement on the composition of the TOC complex, there is still no unanimous acceptance of the 1 MDa TIC complex consisting of Tic20, Tic214, Tic100, and Tic56 as the major TIC complex [2,3,58], although its key role for chloroplast protein import has been demonstrated conclusively not only for *Arabidopsis* but also for the green alga *Chlamydomonas* [14,18]. The classical model proposed Tic110 and Tic40 together with Tic20 as the core components of TIC. However, if one accepts this model, it is rather surprising that Tic110 and Tic40 were not detected in significant amounts when the complex was co-immunoprecipitated with translocating preproteins or with Tic 20 in *Arabidopsis* and *Chlamydomonas* [14,18]. It is clear that both Tic110 and Tic40 play an essential role in chloroplast biogenesis as their null mutants are embryo-lethal. One possibility is that they act further downstream of the translocon channel. It is also clear that there must be at least one additional chloroplast protein import system based on the genetic analysis of single and double mutants deficient in translocon components, which may include Tic20-IV in *Arabidopsis*. A similar protein import machinery may be operational in grasses because they lack the 1 MDa TIC complex and the Ycf2-FtsHi complex.

The new assignment of the Ycf2-FtsHi complex as a chloroplast import motor has also raised some controversy in the field as the earlier reports indicated that Hsp93/ClpC, cpHsp70, and Hsp90C are components of the import motor [3,58]. The exact roles of these chaperones need to be clarified further. Here, also these two models could be reconciled by assuming the existence of two distinct protein import systems consisting of different TOC, TIC, and import motors, which may have the different client and developmental specificities (Fig. 1).

An important novel aspect of the chloroplast protein import system bears on its regulation by UPS as the protein flux through TOC/TIC varies significantly during development and in response to specific environmental signals [59]. UPS acts at different levels. It

selectively degrades transcription factors regulating nuclear genes of chloroplast proteins, removes unimported chloroplast precursor proteins in the cytosol, and reconfigures the TOC complex in response to developmental and environmental cues. Only a few molecular players have been identified, and much remains to be discovered in this complex regulatory network, which coordinates the UPS with the chloroplast proteolytic system to maintain proteostasis in the chloroplast through modulation of the protein import apparatus.

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## Conflict of interest

The authors declare no conflict of interest.

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