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Review Article

A proteostasis network safeguards the chloroplast proteome

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Several protein homeostasis (proteostasis) pathways safeguard the integrity of thousands of proteins that localize in plant chloroplasts, the indispensable organelles that perform photosynthesis, produce metabolites, and sense environmental stimuli. In this review, we discuss the latest efforts directed to define the molecular process by which proteins are imported and sorted into the chloroplast. Moreover, we describe the recently elucidated protein folding and degradation pathways that modulate the levels and activities of chloroplast proteins. We also discuss the links between the accumulation of misfolded proteins and the activation of signalling pathways that cope with folding stress within the organelle. Finally, we propose new research directions that would help to elucidate novel molecular mechanisms to maintain chloroplast proteostasis.

The proteostasis network

Components of the protein homeostasis (proteostasis) network have the crucial task of preserving proteins in their native (correctly folded) conformations, even under challenging and stressful cellular conditions that cause protein misfolding and aggregation. Most proteins must be targeted to distinct subcellular localizations and fold into well-defined 3D structures to perform their biological function [1]. Proteostasis mechanisms include regulated protein translation, protein folding by molecular chaperones, and protein degradation pathways such as autophagy and proteasome-mediated degradation [2,3] (Figure 1). Proteostasis must be maintained in the cytosol and organelles to assure proper cell function. In this review, we will focus on proteostasis mechanisms that safeguard the integrity of thousands of proteins that localize in plant chloroplasts, indispensable organelles for photosynthesis, production of metabolites, and sensors of environmental stimulus.

Chloroplast proteins are imported and sorted into the chloroplast

Chloroplasts and other plastids were originated through endosymbiosis when a eukaryotic host engulfed a photosynthetic prokaryote. Over time, many prokaryotic genes were relocated to host nuclear genome and chloroplasts retained a small plastid genome that contains about 100–250 genes [4]. Hence, chloroplasts depend on thousands of proteins that are encoded by the nuclear genome and must be imported into such organelle [5]. Nuclear-encoded chloroplast proteins are synthesized at cytosolic ribosomes as chloroplast precursors proteins (preproteins) and delivered to the chloroplast surface by heat shock protein (HSP) 70 and HSP90 chaperones. Preproteins contain an unstructured/unfolded N-terminal transit peptide [6] that facilitates their recognition by chaperones and the translocon complexes at the outer (TOC) and inner (TIC) chloroplast envelope membranes [7] (Figure 2). Due to the small pore size of translocon complexes, preproteins are imported unfolded. Once imported into the chloroplast, preproteins are subjected to proteolytic processing, where the transit peptide is cleaved by the stromal

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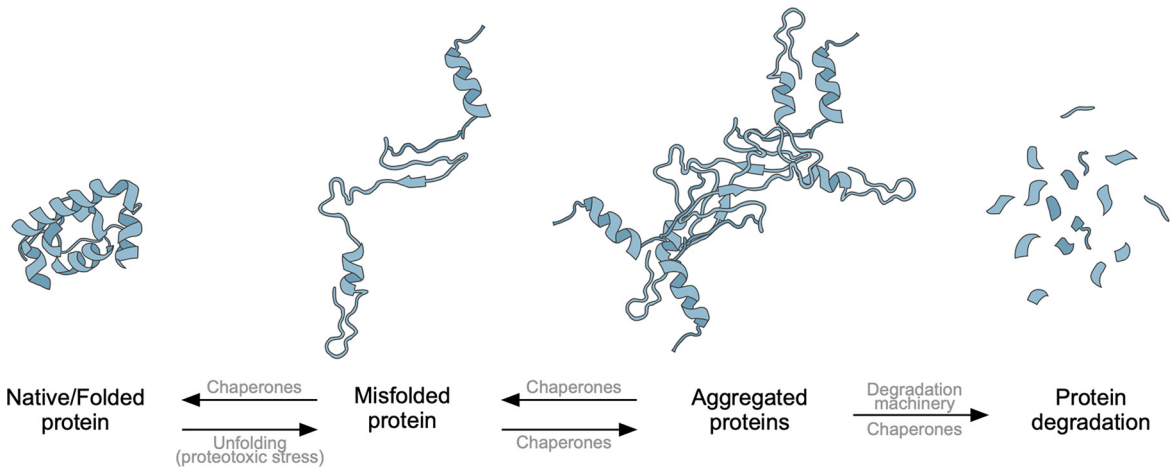

Figure 1. Proteostasis network controls conformation and levels of proteins

Proteins being released out from the ribosome adopt a defined three-dimensional structure, the native or folded state. During proteotoxic stress, proteins misfold (or unfold), tending to self-assemble and forming aggregates that could be targeted to refolding or degradation by protein quality control machinery.

processing peptidase (SPP) [8] (Figure 2). The mature cleaved chloroplast protein is now folded or sorted, with the help of chaperones, into intraorganellar structures such as the thylakoids [9]. A recent study has revealed an important role of condensates in co-ordinating the migration of imported proteins into the thylakoid membranes. Two plant-specific ankyrin-repeat proteins, STT1 and STT2, specifically mediate sorting of chloroplast twin arginine translocation (cpTat) pathway proteins to thylakoid membranes. cpTat substrates bind the N-terminal intrinsically disordered regions (IDRs) of the STT complex and induces liquid–liquid phase separation (LLPS), highlighting the importance of phase-separated droplets as a novel mechanism of intrachloroplast cargo sorting [10].

A set of chaperones promotes protein folding in the chloroplast

A plethora of chaperones and assembly factors are required to ensure the correct functioning of the plastidial proteome, including the HSP90, CPN60, HSP100, HSP70, and DNAJ families (Figure 2). HSP90.5 is the only isoform present in plastids and is essential for plant growth and chloroplast biogenesis [11]. The CPN60 chaperonin complex is formed by α and β subunits and mediates protein targeting to the thylakoid membrane [12]. CLPB3 is the only plastidial member of the HSP100 disaggregase family and forms a hexameric complex that maintains substrates correctly folded during heat stress [13]. Two plastidial HSP70 isoforms are functionally redundant and participate in plant development, thermotolerance, and chloroplast protein import [14,15]. Canonical DNAJ proteins act primarily as cochaperones of HSP70. For example, DNAJC20 regulates the isoprenoid biosynthetic pathway [16]. On the other hand, DNAJA5 and DNAJA6 regulate the assembly of iron–sulfur clusters in chloroplasts [17]. However, non-canonical DNAJ proteins (also called DNAJ-related proteins) act as assembly factors in numerous protein–protein interactions in chloroplasts [18]. For instance, certain DNAJ-related proteins are involved in the assembly of FTSZ ring required for plastid division [19], stabilize photosynthetic intermediate complexes [20], or protect carotenoid biosynthesis enzymes from degradation [21]. Although some chaperones can interact with a wide range of substrates, others display high specificity for a single substrate. This chaperone specificity could be engineered to target a selected chloroplast protein to promote its activity and folding status even in the face of stress conditions.

Protease-mediated degradation in chloroplasts

A folded and correctly sorted chloroplast protein could be damaged by several stresses, including high temperatures, high light, and oxidative conditions. Damaged proteins can be refolded by chaperones or removed by chloroplast proteases that have a prokaryotic origin (Figure 2). The most studied proteases for protein turnover in the chloroplast include the ubiquitous AAA+ family of ATPase caseinolytic protease (CLP), filamentation temperature-sensitive H (FTSH), long-filament phenotype (LON) protease, and degradation of periplasmic (DEG) proteases [22]. Whereas

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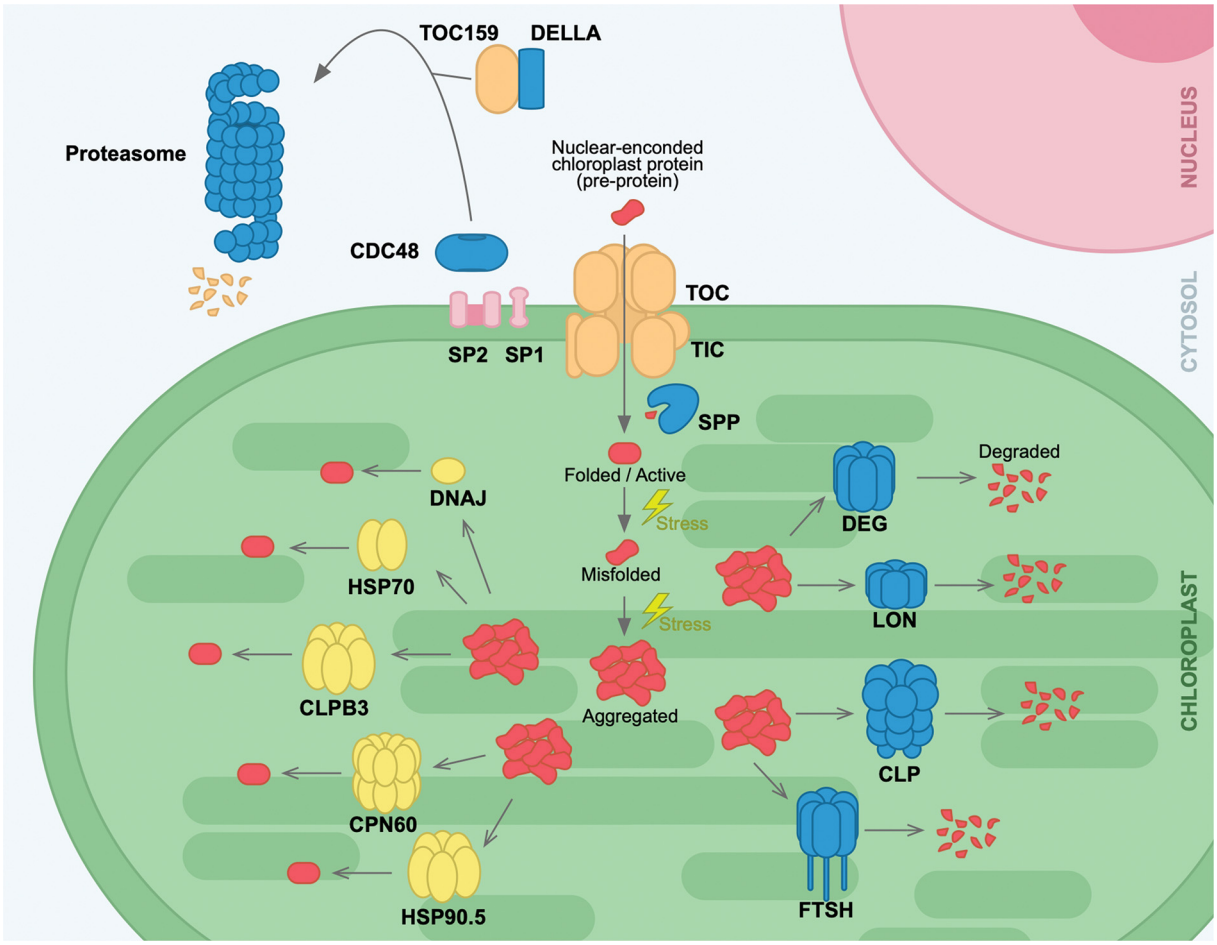

Figure 2. Schematic representation of protein quality control mechanisms in chloroplasts

Chloroplast proteins synthesized in the cytosol are imported into chloroplast through the TOC/TIC translocons complexes with the assistance of chaperones at both sides of the outer envelope membranes (OEMs). SPP cleaves chloroplast transit peptides, and proteins acquire folded/active configuration. However, upon stress conditions such as high temperatures or oxidative stress, proteins misfold and aggregate. Damaged proteins need to be refolded by the action of chaperones (e.g. DNAJ, HSP70, CLPB3, CPN60, and HSP90.5) or degraded by specific proteases (e.g. DEG, LON, CLP, and FTSH). In addition, the CHLORAD system composed of SP1 E3 ligase, SP2 channel protein, and CDC48 chaperone targets OEM proteins for degradation by the proteasome in the cytosol. DELLA proteins also regulate the degradation of TOC159 in a SP1-independent manner.

CLP regulates the level of proteins involved in DNA and RNA maintenance and plastidial metabolism [23,24]; FTSH and DEG proteases regulate the turnover of subunits of photosynthetic complexes [25,26]. Less information is available about the function and targets of LON protease in plastids. Notably, LON1 and LON4 isoforms are targeted to mitochondria and chloroplast [27,28]. Interestingly, these prokaryotic-type proteases share similar features with those present in other organelles from endosymbiotic origin such as apicoplasts and mitochondria [29,30]. Recent studies have unveiled a synergistic role between chaperones and proteases to clear damaged proteins. Under stress situations, proteases and chaperones degrade misfolded proteins avoiding the accumulation of toxic aggregates. For instance, HSP70 co-operates with CLP protease to deliver specific substrates for degradation [49]. Remarkably, the recent elucidation of the structure of the chloroplast CLP complex by cryoelectron microscopy in *Chlamydomonas reinhardtii* revealed that CPN60 chaperone forms a cap that represses CLP proteolytic activity, hence regulating protein folding and degradation [31]. Possibly, the CPN60 complex acts as an adaptor of CLP-delivering substrates. Moreover, CPN60 was also proposed to interact with FTSH11 [32], indicating that CPN60 may also have a role in protein degradation. Therefore, it is likely that other protease-chaperone interactions might be necessary to regulate protein turnover in the chloroplast.

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Cytosolic-mediated degradation of chloroplast proteins

Besides chloroplast proteases, chloroplast-associated protein degradation (CHLORAD) is a set of molecular mechanisms responsible for the specific degradation of chloroplast OEM proteins in the cytosol [5]. This cellular process involves several proteins including the SP1 E3 ligase that specifically directs the ubiquitination of substrates such as TOC proteins [33]. Subsequently, the channel protein SP2 and the cytosolic chaperone CDC48 co-operate in the retro-translocation of targeted proteins and their delivery to the proteasome for further degradation [34]. In addition to ubiquitin-dependent systems, the small ubiquitin-like modifier (SUMO) system is also involved in the regulation of chloroplast OEM proteins. Reduced SUMOylation stabilizes TOC protein complexes [35]. However, during protoplast to chloroplast transition, SUMOylation stabilizes TOC159 subunit from DELLA-dependent degradation [36]. This mechanism seems to constitute an alternative route to SP1 for targeting substrates to the proteasome [37]. Thus, future characterization of post-translational modifications of OEM proteins and unveiling the targets of CHLORAD, SUMOylation, and DELLA pathways will be essential to better understand the molecular mechanisms that govern their stability. Finally, although ubiquitination may trigger chloroplast autophagy of whole-damaged chloroplasts (chlorophagy), it seems that it is a SP1-independent process [38].

Chloroplast-misfolded proteins activate signalling pathways to release folding stress

Upon proteostasis imbalance, stress signalling pathways increase the levels of chaperones and the activity of proteases-supporting cell homeostasis. These responses restore proteostasis and allow the cytosol or organelles to cope with the overload of unfolded/misfolded proteins. However, it is not known if upon strong or prolonged proteotoxic stress, programmed cell death can be triggered as observed in animal models [39] (Figure 3A). Misfolded proteins are detected by compartment-specific stress response pathways that result in transcriptional responses to ameliorate misfolded protein load. Under certain conditions, chloroplast preproteins can shortly remain in the cytosol and then be recognized by HSC70.4 in the cytosol, ubiquitinated by the CHIP E3 ligase, and degraded in the 26S proteasome [40–42]. However, when chloroplast protein import or proteasome activity is down-regulated or perturbed, unfolded/misfolded preproteins accumulate in the cytosol-eliciting toxic effects in the cell. To counteract proteotoxic stress, the cell activates a signalling pathway known as the cytosolic unfolded protein response (cytUPR) [43,44]. Such response is activated by the heat shock transcription factor (HSF) HSFA2. The expression of *HSFA2* is highly increased shortly after the production of unfolded proteins [45] and activates the expression of target genes such as the *HSP70* and *HSP90* [44,46]. The rapid increase in *HSFA2* after protein folding stress is elegantly regulated. Under normal conditions, class A HSFs (HSFA1D, HSFA7A, and HSFB1) are constitutively expressed as an inactive form bound to HSP90. [47]. However, upon proteotoxic stress, the HSFs-HSP90 complex dissociates and releases the HSFs due to translocation of HSP90 chaperone to misfolded and damaged proteins. In this way, free HSFs activate the rapid expression of *HSFA2* [46]. Then, downstream HSFA2 target genes and other proteostasis-related genes are up-regulated (Figure 3B).

Chloroplast-to-nucleus signalling pathways can also be activated by proteins that after being imported into chloroplasts are damaged, misfolded, or aggregated [48–50]. For instance, when the stromal CLP protease is genetically or chemically impaired, it causes the accumulation of misfolded/unfolded substrates [50–52]. Moreover, the accumulation of damaged PSII core proteins in a mutant affected in the protease FTSH2 induces proteostasis-related genes [53]. Upon chloroplast proteostasis failure, unknown signalling mechanisms detect misfolded or damaged proteins activating a chloroplast unfolded protein response (cpUPR). The cpUPR induces the expression of a specific set of genes to promote chloroplast homeostasis, photosynthesis, and cell survival [50,53]. The activation of the cpUPR appears to be dependent on HSFA2. This transcription factor is rapidly induced after chloroplast-folding stress activating downstream nuclear-encoded genes that code for the chloroplast HSP21 chaperone and the disaggregase CLPB3 (Figure 3B). Consequently, folding capacity is increased and restores the functional integrity of plastid proteins [50,53,54]. Recently, mutant screening in the algae *C. reinhardtii* led to the identification of the cytosolic kinase MARS1 as part of the signalling pathway involved in transmitting the cpUPR signal from the chloroplast to the nucleus [55].

The cpUPR can be considered part of the plastid operational retrograde signalling pathways that controls the operation of the chloroplast in response to changing environmental conditions (operational control) [56]. For instance, rapid fluctuations in the amount of light are perceived in changes in the reduced/oxidized (redox) state of photosynthetic electron transport components in the chloroplast. Such changes generate a signal that modifies nuclear gene expression through HSFs. It was demonstrated that HSFA1D, HSFA2, and HSFA3 regulate *ASCORBATE PEROXIDASE 2* (*APX2*) expression in response to changes in redox status [57]. It is tempting to speculate that light stress could cause protein aggregation and activate APX2 expression. However, it is unknown if the molecular players and

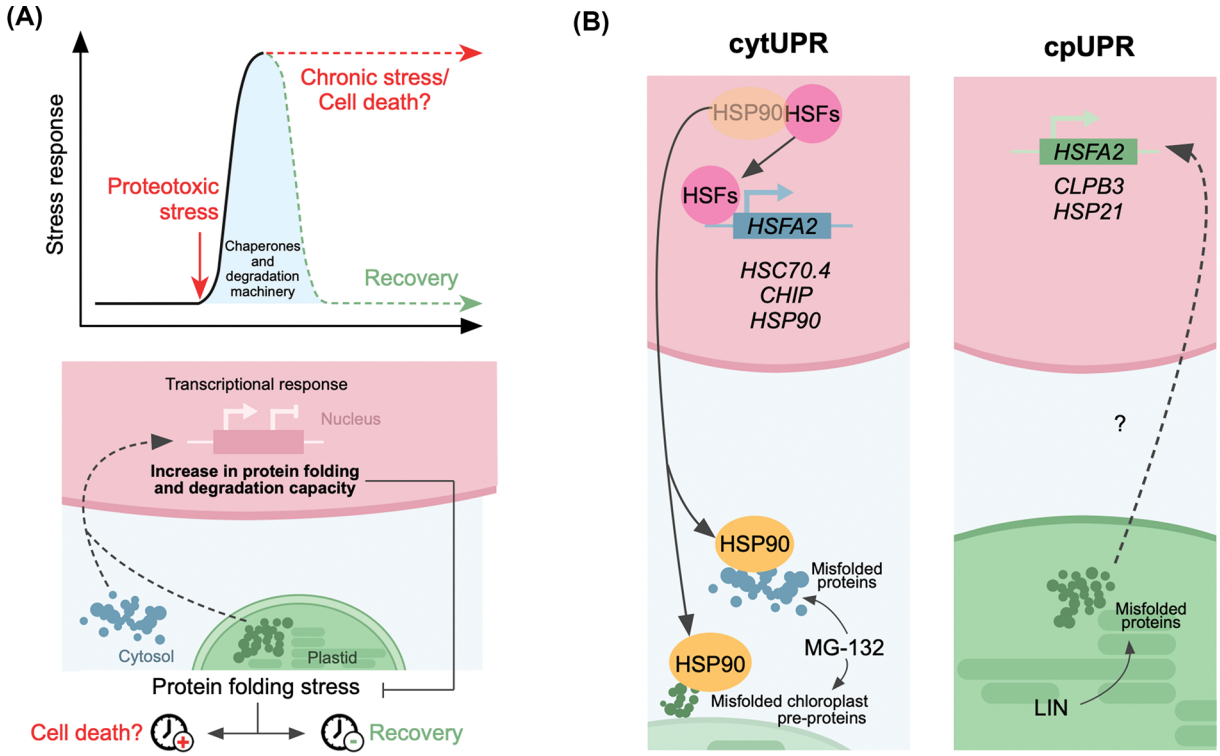

Figure 3. Overview of folding stress responses caused by chloroplast proteins

(**A**) Proteotoxic stress triggers transient prosurvival stress responses that enhance folding and degradation capacity to restore protein homeostasis. However, upon strong or prolonged proteotoxic stress, programmed cell death could be activated. Misfolded proteins are detected by compartment-specific stress response pathways that result in transcriptional responses to ameliorate misfolded protein load. (**B**) In the presence of misfolded proteins (e.g. in the presence of the proteasome inhibitor MG-132), HSF-s-HSP90 complex dissociates, and free HSF activates the expression of *HSFA2*, activating the cytUPR to restore proteostasis. In the cpUPR, damaged/misfolded proteins can be sensed by unknown mechanisms. The chloroplast translation inhibitor Lincomycin (LIN) can cause protein aggregation unleashing the cpUPR.

signals involved in the plant cpUPR and other plastid operational retrograde signalling pathways are connected. The design of genetic screenings in Arabidopsis aiming to block the specific nuclear response triggered upon disruption of chloroplast proteostasis will help to identify proteins involved in chloroplast-to-nucleus retrograde signalling and cpUPR modulation.

Future research directions in chloroplasts proteostasis

Cross-talk between proteostasis and retrograde signalling pathways

Impairment of plastidial proteases such as CLP and FTSH activates the cpUPR due to the accumulation of aggregated proteins (Figure 3B) [50,53,58]. Furthermore, several additional retrograde signalling pathways rely on the production of specific metabolic signals, reactive oxygen species (ROS), and translocation of retrograde signalling proteins [59,60]. Currently, there is virtually no information about the cross-talk of the cpUPR with these alternative signalling pathways, and only a few presumed interactions have been implied. For instance, EXECUTER1 protein is an essential sensor for singlet oxygen, a ROS byproduct of defective photosystem II activity. Executer localizes at the grana margins in chloroplasts where singlet oxygen is generated under high light stress [61]. Strikingly, singlet oxygen signalling depends also on FTSH2 since this protease regulates the turnover of EXECUTER1 [62]. Concomitantly, EXECUTER1 is highly enriched among the interactors of the CLPC1 adaptor subunit part of the CLP complex [24,63]. However, it is unclear whether EXECUTER1 modulates the activity of CLP. It could be possible that EXECUTER1 is a degradation target of both FTSH and CLP. Regardless, future efforts should be aimed to elucidate whether singlet oxygen production may be part of the cpUPR signalling cascade. Moreover, ROS production also promotes the accumulation

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of aggregated proteins [64]. Therefore, it is important to understand if these signalling pathways (ROS and cpUPR) are interconnected.

A link between chloroplast protein import and cpUPR has been recently reported. The disruption of chloroplast protein import in algae unleashes a stress response that results in the up-regulation of a set of proteins that match well those genes whose transcript expression is increased upon CLP protease depletion [55,65]. Future experiments should be carried out to compare the gene expression profiles between plants lacking either defects on proteases or on protein import components.

Finally, another important component of chloroplast-to-nucleus communication is GUN1, a central integrator of biogenic retrograde signals involved in the down-regulation of the expression of photosynthetic genes upon stress conditions [66,67]. Interestingly, GUN1 interacts with several components of the proteostasis machinery in chloroplasts, including chaperone HSP70 and CLP protease [68], and a later study demonstrated that indeed CLP regulates GUN1 turnover in plastids [69]. In addition, GUN1 also facilitates the import of nuclear-encoded plastid proteins by physically interacting with HSP70 [43,70]. Together, current data indicate that GUN1 is an important proteostasis regulator in chloroplasts. However, its specific function and mechanism of action remain to be elucidated. Even though GUN1 does not activate *HSFA2* expression in the cpUPR [50], future work should be made to determine whether GUN1 plays a role in the cpUPR.

Translocation of misfolded proteins between cytosol and chloroplasts

Termination of toxic protein aggregates is important for organismal survival. Interestingly, a recent study unveiled a novel mitochondria-mediated cytosolic proteostasis mechanism. It was demonstrated that cytosolic aggregate-prone proteins engage with mitochondrial import machinery on the outer membrane and enter the mitochondria for further degradation [71]. It is tempting to speculate that plant chloroplasts could also degrade cytosolic aggregated proteins through their import and degradation within chloroplasts. In agreement with this hypothesis, proteome analysis of chloroplast thylakoid fractions depleted of the FTSH protease under heat stress identified several extraplastidial cytosolic proteins. However, it is unclear how cytosolic proteins found in thylakoid fractions of heat-treated plants lacking FTSH could be relocated into the chloroplast [72]. Future efforts must be made to unveil if chloroplasts are capable of importing misfolded cytosolic proteins for further degradation. On the other hand, pioneer works in nonplant models have shown that mitochondrial-associated degradation (MAD) is responsible for the clearance not only for outer mitochondrial membrane (OMM) proteins but also for inner membrane space (IMS) proteins. Such mechanism is mediated through the translocase of the outer mitochondrial membrane (TOM) complex [73,74]. It has been recently shown that intrachloroplast proteins are exported to the cytosol and degraded by the proteasome. CDC48 is involved in the degradation of plastid-encoded proteins RBCL and ATPB by the proteasome [75]. Additional studies must be directed to unveil if additional chloroplast-damaged proteins are retro-translocated through the channel protein SP2 and the cytosolic chaperone CDC48 for further cytosolic degradation or if CDC48 co-operates with the chlorophagy machinery. However, it will be challenging to differentiate between proteasomal degradation of retro-translocated chloroplast proteins and degradation of unimported preproteins that accumulate when chloroplast protein import is disrupted.

Phase separation and chloroplast proteostasis

Some proteins condensate into a dense phase that often resembles to liquid droplets in a process known as phase separation. Such process depends on the concentration and identity of the protein and on environmental conditions including temperature, cosolutes, pH, etc. Thus, some proteins undergo stimulus-responsive phase separation [76]. Recent studies have revealed that some plant prion-like proteins or intrinsically disordered proteins (IDPs), liquid–liquid phase separate and function as sensors to monitor temperature and water availability, respectively [77,78]. As we mentioned above, it was recently characterized the importance of LLPS to facilitate cargo targeting and translocation across thylakoid membranes [10]. Notably, the formation of stress granules also involves LLPS. Stress granules are nonmembrane compartments that sequester and organize biomolecules, proteins, nucleic acids, and metabolites in response to stress [79]. Plant stress granules were first studied and characterized in the cytosol [80]; however, they were recently characterized within the chloroplast [81]. Similar to the cytosolic stress granules, the plastid stress granules assemble rapidly in response to stress and disappear when the stress ceases. Plastid stress granules are composed of a stable core and a fluid outer shell. Such nonmembrane structures sequester proteins including RNA-binding protein with prion-like domains, ATPases, chaperones, and amino acids. Moreover, plastid stress granules also sequester the complete magnesium chelatase complex, which is involved in photosynthetic acclimation to stress. The composition and dynamics of the plastid stress granules strongly suggest that they have a key role in plant stress tolerance.

The formation of stress granules could be a mechanism to avoid the irreversible aggregation of plastid proteins. For instance, during stress situations, the translation elongation factor Tu (RABE1B) and RUBISCO ACTIVASE are sequestered in stress granules to modulate its activity and avoid its irreversible aggregation [81]. Controlled formation of stress granules or condensates could help to protect specific proteins from stress situations and generating plants with enhanced proteostasis.

Conclusion

Several protein quality control mechanisms tightly control the life of a chloroplast protein. Most chloroplast proteins are encoded in the nucleus, synthesized in the cytosol, and imported to the chloroplast by chaperones and protein import machinery. Once in the chloroplast, proteins perform their functions. However, stress conditions damage the integrity of the proteins promoting misfolding and aggregation. To face folding stress, chloroplasts employ several strategies to avoid the toxic accumulation of misfolded proteins, such as refolding and disaggregation by chaperones, proteolytic degradation, and the controlled formation of stress granules. The molecular characterization of new proteostasis pathways will help engineer crops with stress-tolerant chloroplasts and improve their yield even under adverse conditions caused by climate change.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

APX2, ASCORBATE PEROXIDASE 2; CHLORAD, chloroplast-associated protein degradation; CLP, caseinolytic protease; cpTat, chloroplast twin arginine translocation; cpUPR, chloroplast unfolded protein response; cytUPR, cytosolic unfolded protein response; DEG, degradation of periplasmic; FTSH, filamentation temperature-sensitive H; HSF, heat shock transcription factor; HSP, heat shock protein; IDP, intrinsically disordered proteins; IDR, intrinsically disordered regions; IMS, inner membrane space; LON, long-filament phenotype; MAD, mitochondrial-associated degradation; OEM, outer envelope membranes; OMM, outer mitochondrial membrane; ROS, reactive oxygen species; SSP, stromal processing peptidase; SUMO, small ubiquitin-like modifier; TIC, translocon at the inner envelope membrane of chloroplasts; TOC, translocon at the outer envelope membrane of chloroplasts; TOM, translocase of the outer mitochondrial membrane.

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