Chloroplast Proteases: Updates on Proteolysis within and across Suborganellar Compartments[1][OPEN]

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Chloroplasts originated from the endosymbiosis of ancestral cyanobacteria and maintain transcription and translation machineries for around 100 proteins. Most endosymbiont genes, however, have been transferred to the host nucleus, and the majority of the chloroplast proteome is composed of nucleus-encoded proteins that are biosynthesized in the cytosol and then imported into chloroplasts. How chloroplasts and the nucleus communicate to control the plastid proteome remains an important question. Protein-degrading machineries play key roles in chloroplast proteome biogenesis, remodeling, and maintenance. Research in the past few decades has revealed more than 20 chloroplast proteases, which are localized to specific suborganellar locations. In particular, two energy-dependent processive proteases of bacterial origin, Clp and FtsH, are central to protein homeostasis. Processing endopeptidases such as stromal processing peptidase and thylakoidal processing peptidase are involved in the maturation of precursor proteins imported into chloroplasts by cleaving off the amino-terminal transit peptides. Presequence peptidases and organellar oligopeptidase subsequently degrade the cleaved targeting peptides. Recent findings have indicated that not only intraplastidic but also extraplastidic processive protein-degrading systems participate in the regulation and quality control of protein translocation across the envelopes. In this review, we summarize current knowledge of the major chloroplast proteases in terms of type, suborganellar localization, and diversification. We present details of these degradation processes as case studies according to suborganellar compartment (envelope, stroma, and thylakoids). Key questions and future directions in this field are discussed.

Over 1 billion years of plastid evolution since the endosymbiosis of ancestral cyanobacteria (Douzery et al., 2004), chloroplast biogenesis has gained complexity, with large sets of the endosymbiont genes being transferred to host nuclear genomes. While only 100 endosymbiont genes remain in the plastid genome, with the corresponding proteins biosynthesized there, the nuclear genes have often gained complexity by duplication and diversification of the original endosymbiotic genes. This complexity raises numerous questions regarding (1) at what level gene expression is coordinately controlled, (2) what molecules coordinate the cross talk between chloroplasts and the nucleus, (3) how proteins get across membranes and become imported into chloroplasts, and (4) how the stoichiometries of nucleus- and chloroplast-encoded subunits within individual chloroplast protein complexes such as photosystems and Rubisco are strictly maintained. Given these questions, chloroplast biogenesis has remained a central subject in plant physiology for the last few decades (Jarvis and López-Juez, 2013). In our view, the aforementioned questions point to the importance of protein homeostasis and posttranslational modification, in which proteases play a dominant role. Therefore, we focus on the major events of proteolysis governed by chloroplast proteases, paying particular

ADVANCES

- The 20 known chloroplast proteases include two types, processive proteases and processing peptidases, that shape the chloroplast proteome.
- The major processive proteases have heteromeric structures with unfolding and proteolytic domains and are involved in chloroplast proteome homeostasis by degrading unnecessary proteins.
- Recent reports implicate the extraplastidic ubiquitin proteasomal system and intraplastidic prokaryotic-like proteases in regulation of the chloroplast proteome through the control of protein translocation across the envelopes.
- Stromal proteases function in sequential preprotein maturation, environment-dependent metabolic regulation, and mineral homeostasis; thylakoid proteases are involved in protein maturation and quality control of photosynthetic complexes.

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attention to what kinds of proteases are present and how they exert their functions in chloroplasts.

In this review, we describe the major protease machineries and their intraorganellar functions in proteolytic regulation of the intraplastid proteome landscape, the latter of which is addressed as three case studies covering the different chloroplast compartments (envelope, stroma, and thylakoids). More detailed descriptions of each chloroplastic protease can be found in previous reviews (Kato and Sakamoto, 2010; Clarke, 2012; Teixeira and Glaser, 2013; Kmiec et al., 2014; Adam, 2015; Nishimura and van Wijk, 2015; van Wijk, 2015). In particular, readers are encouraged to refer to the recent excellent review by van Wijk (2015), in which the regulation of proteolytic machineries is thoroughly described, not only in plastids but also in mitochondria and peroxisomes.

OVERVIEW OF THE MAJOR PROTEASES IN CHLOROPLASTS

More than 20 proteolytic machineries have been identified in chloroplasts through biochemical, genetic, bioinformatic, and proteomic approaches during the last two decades (van Wijk, 2015). Figure 1 illustrates our current understanding of chloroplast proteases. Most chloroplast proteases originated from respective bacterial prototypes. During plastid evolution, they have been duplicated and diversified in terms of structure and function (Box 1). Many are metalloproteases or Ser proteases, and a few are Asp proteases. Genetic studies of model plants have demonstrated the physiological importance of these proteases (for review, see Kato and Sakamoto, 2010). Due to space constraints, we outline only the major intraplastid proteolytic machineries, with emphasis on two major processive proteases, Clp and FtsH.

Processive Proteases Clp and FtsH

Clp in the stroma and FtsH on thylakoid membranes are the major conserved ATP-dependent multimeric protease complexes catalyzing processive degradation in their respective suborganellar compartments (Box 2). Chloroplast Clp and FtsH both have two characteristic domains of bacterial origin, namely an AAA+ (for ATPase associated with various cellular activities) chaperone domain for substrate recognition and unfolding and a proteolytic domain for degradation, which are separated into individual subcomplexes for Clp or are organized together within a single protomer for FtsH. In the course of the evolution of photosynthetic organisms, these proteolytic enzymes have been converted from homomeric to heteromeric macromolecular complexes. Despite the complicated heteromultimeric compositions

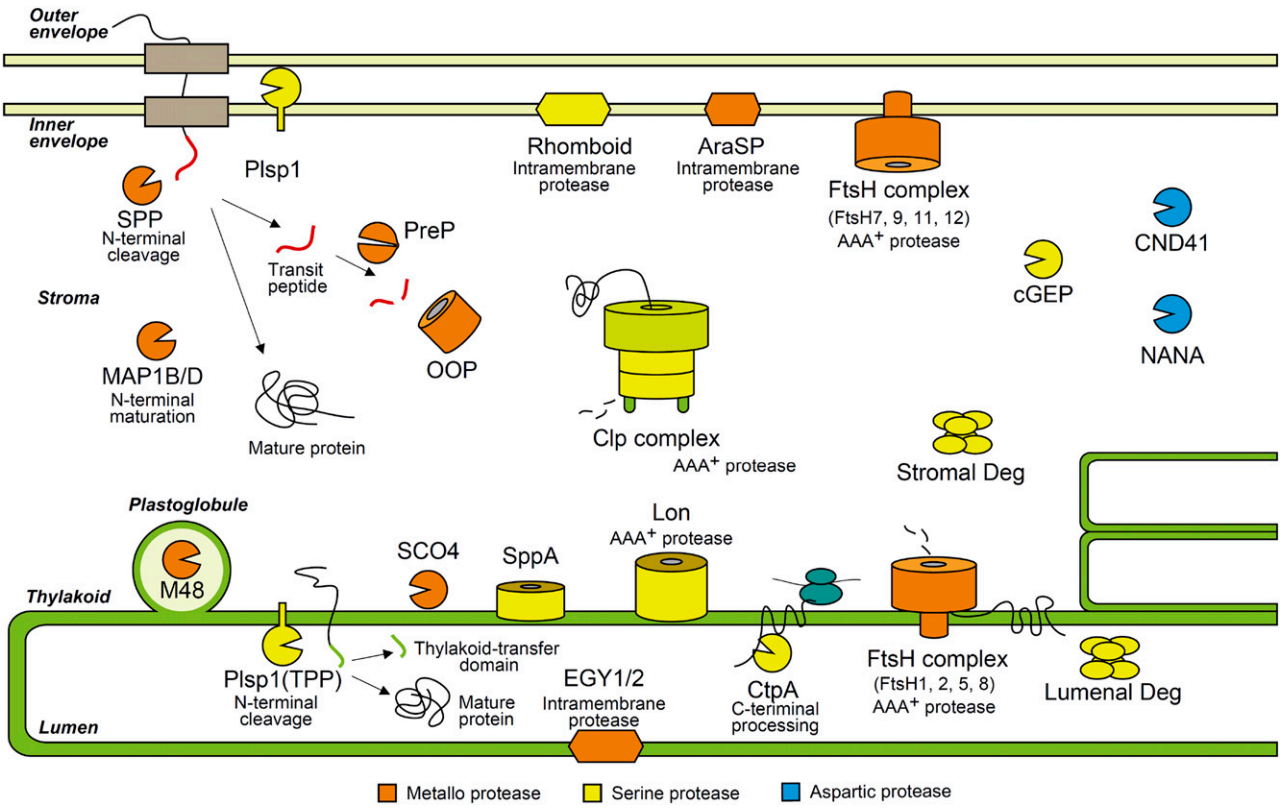


Figure 1. Intraplastid proteases in land plants. Land plant chloroplasts contain three types of proteolytic machines, namely metalloproteases, Ser proteases, and Asp proteases, which are colored in orange, yellow, and blue, respectively.

Box 1. Evolutionary Diversification of Proteases in Photosynthetic Organisms

Chloroplast proteases appear to have diverged into multiple isoforms during the evolution of photosynthetic organisms. It is noteworthy that primitive protease machineries in proteobacteria, like those in *Escherichia coli*, are present as homooligomeric assemblies. By contrast, the cyanobacterial proteases have gained multiple paralogous components, leading to the emergence of heterooligomeric structures. The complexity of protease organization has much increased in land plants. Representative examples include chloroplast FtsH, Deg, and Clps (see table below). The FtsH diversification has produced distinct heterohexameric protease complexes each localized at different intraplastid compartments. The chloroplast Deg proteases have acquired quite diverse domain organizations and function at specific suborganellar locations. The evolution of nonphotosynthetic organisms into photosynthetic organisms has been accompanied by the occurrence of a proteolytically inactive subunit in the stromal Clp core, although the physiological relevance of this is not known. Furthermore, plastid- or plant-specific Clp components have been generated through endosymbiosis. These structural complexities and heterogeneities represent critically important but difficult aspects of chloroplast protease studies. Structural diversification is related to specific functions and, thus, increased physiological importance. For instance, plastid Clp is essential for plant survival, growth, and development, while the proteobacterial prototype is nonessential. FtsH, Deg, and Clps all have gained crucial roles in chloroplast biogenesis and differentiation.

Box 1 Table. *Multiplication of the prokaryotic-type protease subunits through evolution*

The numbers of protease subunits are indicated. For Clps, their chaperones, adaptors, and accessary proteins are not counted.

Protease	Proteobacteria (*Escherichia coli*)	Cyanobacteria (*Synechocystis* PCC 6803)	Chloroplast (Arabidopsis)
FtsH	1	4	9
Deg	1	3	5
Clp	1	4[a]	9[b]

[a]A proteolytically inactive core subunit is included. [b]Four catalytically inactive core components are included.

of these two enzyme complexes, their basic characteristics are well studied, as described below. Another ATP-dependent Ser protease is long filament phenotype (Lon), which is formed by six to seven protomers, each comprising an AAA domain and a protease domain with a catalytic SK dyad (Rotanova et al., 2004). Arabidopsis (*Arabidopsis thaliana*) possesses four Lon proteins, one of which, LON4, is found in the thylakoid membrane, although its precise function in chloroplasts has not been elucidated (Ostersetzer et al., 2007).

Clp

Clp is an ATP-dependent Ser-type protease complex that comprises a chaperone subcomplex and a tetradecameric proteolytic core. The chaperone complex is composed of ClpC1/C2/D, each presumably constituting homooligomers, with ClpC1 as the major chaperone. The ClpC/D structure consists of an N-terminal region called the N domain as a substrate/adaptor docking site, two ATPase domains involved in substrate unfolding and translocation into the core, and an IGF motif (for Ile-Gly-Phe) and an R motif (named for its characteristic Arg residue), both required for core docking, with ClpC but not ClpD containing a uvrB/C motif of unassigned function (Nishimura and van Wijk, 2015). ClpC/D can recognize substrates directly (Rosano et al., 2011; Bruch et al., 2012; Huang et al., 2016), although recognition of a subset of proteins is mediated by a dedicated binary adaptor consisting of ClpS1 and ClpF (Nishimura et al., 2013, 2015). The basic ClpS structure has an N-terminal extension for substrate delivery to the chaperone and a C-terminal core domain for substrate recognition and chaperone binding (Dougan et al., 2002; Zeth et al., 2002; Erbse et al., 2006; Rivera-Rivera et al., 2014). ClpF is a plastid-specific ClpS1-interacting protein with a tripartite structure harboring a unique N-terminal domain for adaptor-substrate-chaperone binding, a uvrB/C motif for chaperone interaction, and a YccV-like domain of unknown function, the last two of which likely are derived from two distinct proteins of bacterial origin (Nishimura et al., 2015). The ClpS prototype functions in the N-end rule pathway, in which the half-life of a protein correlates with the identity of its N-terminal amino acid (Erbse et al., 2006; Varshavsky, 2011). The presence of another substrate recognition and delivery mechanism involving the binary adaptor in chloroplasts has been suggested (Nishimura et al., 2015).

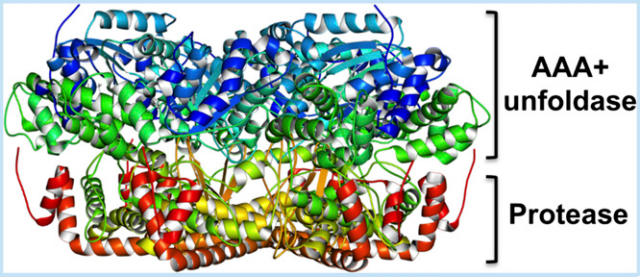
The Clp core consists of two asymmetric rings, namely the P ring containing catalytic subunits ClpP3/P4/P5/P6 in a 1:2:3:1 ratio and the R ring containing proteolytically active ClpP1 (the only chloroplast-encoded subunit) and proteolytically inactive ClpR1/R2/R3/R4 proteins in a 3:1:1:1:1 ratio (Olinares et al., 2011). Clp core assembly and stabilization require plant-specific accessory proteins ClpT1/2 (Peltier et al., 2004; Sjögren and Clarke, 2011; Clarke, 2012; Kim et al., 2015). Loss-of-function mutants for the ClpC1 chaperone and the ClpPRT core show pale-green, seedling-lethal, or embryo-defective phenotypes, whereas knockouts for two adaptor proteins and ClpC2/D display no visible effects, underscoring their distinct contributions to plant development.

FtsH

FtsH is a membrane-associated ATP-dependent zinc metalloprotease complex whose protomer consists of an AAA domain and a proteolytic domain containing a metal-binding H-E-x-x-H motif (Ito and Akiyama, 2005). Land plants possess multiple FtsH orthologs. Nine out of 12 Arabidopsis FtsH homologs are found in the chloroplast, with the four major isoforms (FtsH1/2/5/8) all thylakoid localized via their single transmembrane domains. Interchangeability and functional redundancy have been observed between FtsH5 and

Box 2. Architecture and Action of the Processive Proteases

Three bacterial-like ATP-hydrolyzing protein-degrading enzymes, Clp, FtsH, and Lon, catalyze processive degradation. Their architecture consists of the AAA+ chaperone ring and the barrel-shaped protease complexes (see fig. below). The catalytic center is positioned inside the proteolytic chamber so as to avoid nonspecific degradation of proteins. Substrate access to the protease domain is governed by the chaperone machinery that directly recognizes the protein substrate. The protein is then unfolded and translocated continuously in an ATP-dependent manner to and inside the protease chamber, where it undergoes endoproteolytic cleavage. This chaperone-mediated energy-dependent continuous supply of the substrate proteins allows for targeted processive proteolysis. In Clp, the chaperone and the protease domains are separated into two different assemblies, and small peptide fragments can pass directly through a narrow entrance pore to the protease chamber, where they are degraded. However, folded proteins are unable to enter the pore, and substrate access is controlled at the chaperone gate. Such substrate access control and degradation mechanisms also are found for the cytosolic ubiquitin-dependent proteasomal degradation systems.



Box 2 Figure. Stucture of the archetypal processive protease. Side view of the soluble domain structure of the hexameric FtsH protease from a thermophilic bacterium *Thermus thermophiles* is shown as a representative structure of the processive protease. Its characteristic AAA+ unfoldase chaperone and protease domains are indicated. The image of the FtsH structure (PDB ID: 4EIW) was obtained from the Protein Data Bank of Japan (PDBj).

FtsH1 (type A) and between FtsH2 and FtsH8 (type B; Yu et al., 2004, 2005; Zaltsman et al., 2005), suggesting that FtsH exists as a hexameric heterocomplex with two types of isoforms. The stoichiometry of type A to type B subunits in an FtsH complex is estimated as 2:4 (Moldavski et al., 2012), whereas the cyanobacterial prototype comprises type A and B subunits in a 3:3 ratio with an alternating arrangement (Boehm et al., 2012). FtsH2 and FtsH5 are targeted to the thylakoid via the TAT (for twin-Arg translocation) pathway and the SEC (for secretion) pathway, respectively (Rodrigues et al., 2011). Leaf variegation phenotypes are observed in mutants for FtsH2 (also known as YELLOW VARIEGATED2 [VAR2]) and FtsH5 (VAR2; Fig. 2). FtsH2 and FtsH5 are the most abundant and the second most abundant FtsH isomers, respectively, reflecting the severity of their loss-of-function phenotypes of leaf variegation compared with the two other minor subunits, whose knockouts show wild-type-like appearance (Sakamoto et al., 2003; Zhang et al., 2010). ROS, including superoxide radicals and hydrogen peroxide (the former of which is shown in Fig. 2), are detected in the green but not the white leaf areas, specifically in the chloroplasts, of the *var2* mutant grown even under normal light conditions, indicative of its persistent photooxidative stress (Kato et al., 2009). A series of genetic studies in Arabidopsis identified trans-acting factors suppressing *var1/var2* leaf variegation; these included Clp subunits, translation factors, a pentatricopeptide repeat protein, a pseudouridine synthase homolog, a plastid transcriptionally active chromosome component, a prokaryotic-like peptide deformylase, ribosomal proteins, circularly permuted GTPase family proteins, and a sigma factor (Park and Rodermel, 2004; Miura et al., 2007; Yu et al., 2008, 2011; Liu et al., 2010a, 2010b, 2013; Adam et al., 2011; Wu et al., 2013; Powikrowska et al., 2014; Hu et al., 2015; Ma et al., 2015; Qi et al., 2016). Several models have been proposed to explain leaf variegation suppression, but the precise mechanism remains elusive (Miura et al., 2007; Yu et al., 2008).

Another set of FtsH isomers, FtsH7/9/11/12, is present in the envelope (Fig. 1; Wagner et al., 2012). FtsH7/9 share high sequence similarity and, therefore, have been speculated to constitute a heteromeric protease complex. FtsH11 is involved in high-temperature tolerance, which is reminiscent of its bacterial prototype (Chen et al., 2006, 2007). High-light responses in the *ftsh11* mutant are normal, unlike the variegated mutants defective in FtsH2/5. FtsH11 is a potential target for an intramembrane protein degradation pathway (Knopf et al., 2012). FtsH11 also is localized in mitochondrial inner membranes, where it seems to act in parallel with FtsH4 (Urantowka et al., 2005). FtsH11 has a unique N-terminal extension of unknown function. Although the proteolytic domain of mitochondrial FtsH11 faces the inner membrane space, similar to FtsH4 and the yeast ortholog, its membrane topology in the chloroplast is not known.

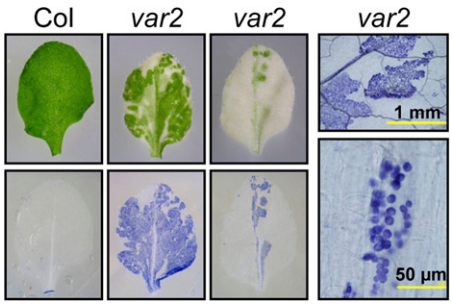


Figure 2. Leaf variegation in the Arabidopsis *var2* mutant lacking FtsH2. Four-week-old wild-type Columbia (Col) and *var2* leaves are shown, along with in situ detection of reactive oxygen species (ROS; superoxide radicals) visualized by NBT staining (blue color). The right two images show closeup views of variegated leaves where ROS is confined to green sectors and chloroplasts. Images are from Kato et al. (2009).

The other chloroplastic isomer, FtsH6, is dispensable for normal growth under high light and natural environmental stress conditions. However, its suborganellar location and precise function remain unclear (Wagner et al., 2012; Lu, 2016).

Endopeptidase Deg Participating in Processive Proteolysis

Deg (originally termed DegP, for degradation of periplasmic proteins; also known as high-temperature requirement A) also is well known to function together with the processive protease FtsH in chloroplasts (see below). Deg is an ATP-independent Ser-type endopeptidase that harbors an N-terminal proteolytic domain with an HDS catalytic triad and C-terminal PDZ (for PSD-95/SAP90, disc large, and ZO-1) domain(s) involved in protein-protein interaction (Schuhmann and Adamska, 2012). Deg1/5/8 and Deg2/7 are present in the lumenal and stromal sides of the thylakoid membrane, respectively (Fig. 1). The number of PDZ domains differs among Degs: there is one in Deg1/8, two in Deg2, four in Deg7, and none in Deg5 (Schuhmann and Adamska, 2012). Monomeric Deg forms a trimer, with each protomer connected via the protease domain, and trimeric units assemble through interactions between the PDZ domains into higher-ordered oligomers, including hexamers (Clausen et al., 2002). For example, Deg1 undergoes a conformational transition from its inert monomer through the trimeric intermediate to the active hexamer upon lumenal acidification (Kley et al., 2011). Deg7 has a characteristic primary structure comprising one active and one degenerated protease domain with four PDZ domains. Its trimerization is mediated by the degenerated protease domains (Schuhmann et al., 2011). Deg2 possesses two PDZ domains (PDZ1 and PDZ2) and forms a hexamer by dimerization of trimers through interactions of the PDZ2 domain with the protease domain and with PDZ1, but the hexamer is quite rigid in structure and rather inactive, whereas a higher-ordered structure is suggested to represent the active state (Sun et al., 2012). Proteolytically inactive Deg5 and proteolytically active Deg8 together constitute a stable heterohexameric complex in a 1:1 ratio (Sun et al., 2007).

Processing Proteases

Most plastid proteins are encoded in the nuclear genome and biosynthesized in the cytosol as preproteins bearing N-terminal transit peptides, followed by translocation across the envelope protein import TIC/TIC channels (Jarvis and López-Juez, 2013). Imported preproteins are subjected to sequential proteolytic processing for transit peptide cleavage and maturation. Many thylakoid proteins, including photosynthetic proteins of the thylakoid membrane, are subsequently sorted to their proper locations through bacterial-like SEC, TAT, and signal recognition particle (SRP) pathways. Their N-terminal bipartite transit peptides, which consist of a thylakoid transfer signal (TTS) following the transit peptide, also are cleaved by limited proteolysis (Celedon and Cline, 2013).

Stromal processing peptidase (SPP) is a metalloendopeptidase that removes transit peptides from preproteins with broader substrate specificity (Richter and Lamppa, 1998). Transit peptide degradation involves one or two isoforms of the presequence peptidase (PreP1/2), which belongs to a metalloendopeptidase family (Stahl et al., 2002; Bhushan et al., 2003, 2005; Moberg et al., 2003). Organellar oligopeptidase (OOP) is another zinc metalloprotease that participates in targeting peptide degradation. OOP degrades peptides with lengths of eight to 23 amino acid residues but fails to act on folded proteins, consistent with its catalytic cavity size, as in the case of PreP1/2 (Kmiec et al., 2013, 2014). The OOP-null mutant phenotype is wild type like, but genetic interaction with the *prep1 prep2* double mutant is observed, indicating complementary functions for OOP and PreP1/2 (Kmiec et al., 2013).

Arabidopsis has three isoforms of the plastidic type I signal peptidase I family, namely Plsp1 and Plsp2A/B (Hsu et al., 2011). Thylakoidal processing peptidase (TPP) is the integral membrane protease with a lumen-facing Ser/Lys-type proteolytic domain, and it cleaves TTSs off preproteins (Chaal et al., 1998). Plsp1 functions as the TPP in maturation of a subset of the SEC/TAT substrates and is necessary for proper thylakoid formation (Endow et al., 2010; Shipman-Roston et al., 2010; Midorikawa et al., 2014). Plsp1 also is targeted to the envelope, where it participates in TOC75 precursor processing (Inoue et al., 2005; Shipman and Inoue, 2009). The biochemical properties and physiological functions of Plsp2A/B are currently unclear.

C-terminal processing protease, a monomeric Ser-type protease that cleaves off a C-terminal extension for maturation of the D1 precursor protein, is another type of processing peptidase in the thylakoid lumen (Anbudurai et al., 1994; Fujita et al., 1995; Oelmüller et al., 1996; Satoh and Yamamoto, 2007; Che et al., 2013). Its basic structure contains a PDZ domain for D1 C-terminal binding and a protease domain with an SK dyad for proteolysis (Liao et al., 2000).

CASE STUDIES OF PROTEOLYTIC REGULATION OF CHLOROPLAST PROTEINS

Following the above overview of the major protease machineries in the chloroplast, we here present three case studies of proteolytic regulatory processes based on their intraorganellar compartments, namely the envelopes (case 1), stroma (case 2), and thylakoid membranes (case 3). In case 1, recently discovered regulation and quality control mechanisms of protein import across the outer and inner envelopes by the actions of extraplastid as well as intraplastid protein degradation machineries are illustrated. Case 2 describes multiple proteolytic events involving the stromal processive protease or the processing peptidases. Examples of

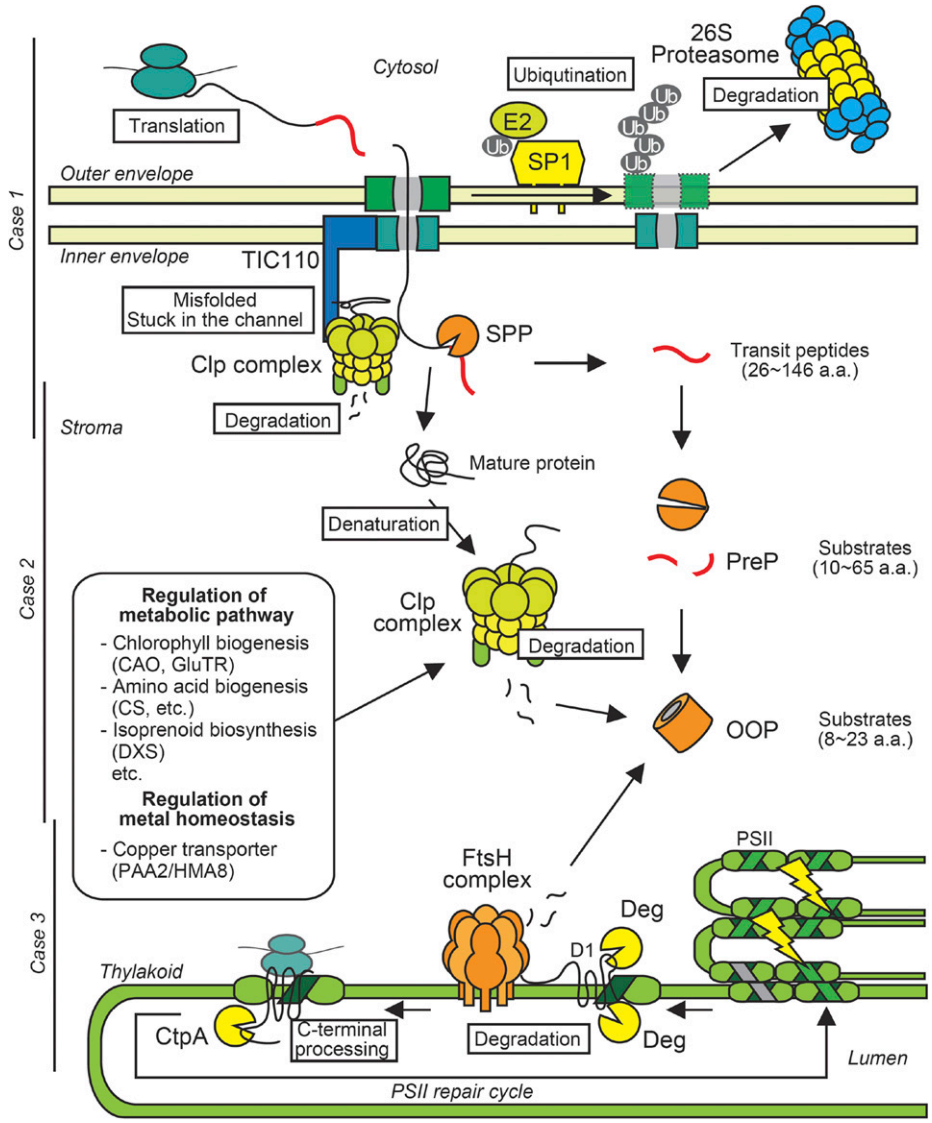


Figure 3. Proteolytic regulation of intraplastid proteome homeostasis. The scheme shows multiple events in the regulation of chloroplast protein homeostasis, which involves not only intraplastidic but also extraplastidic protein degradation machineries. a.a., Amino acids.

environmentally responsive proteolysis also are included. Finally, in case 3, proteolytic functions in thylakoid protein biogenesis, homeostasis, and quality control are exemplified. These proteolytic events are graphically summarized in Figure 3.

Case 1: Regulation and Quality Control of Protein Import across the Envelopes (26S Proteasome and Clp)

Proteolytic Reorganization of Protein Import Machinery by the Cytosolic Ubiquitin-Proteasome System

Jarvis and coworkers demonstrated that the ubiquitin-proteasome system regulates protein import across the chloroplast outer envelope through the degradation of TOC components. Genetic screening for the suppressors of a TOC33 knockout mutant (*plastid protein import1* [*ppi1*]) identified a RING-type ubiquitin E3 ligase, SUPPRESSOR OF PPI1 LOCUS1 (SP1; Ling et al., 2012). SP1 has a cytosolically exposed RING finger domain for ubiquitination and two transmembrane domains for integration into the outer envelope, where it recognizes TOC components, including TOC75, TOC159, and TOC33, through its intermembrane space domain. SP1 ubiquitinates these client proteins for proteasome-mediated proteolysis (Fig. 3). The TOC complexes use different receptor isoforms for the recognition of distinct sets of preproteins (Paila et al., 2015). Therefore, SP1 functions in plastid proteome remodeling through reorganization of the TOC constituents, thereby regulating organellar differentiation in response to environmental context. The *sp1* mutant shows inefficient deetiolation upon illumination and delayed chloroplast-to-gerontoplast transition during dark-induced senescence, whereas the SP1 overexpressor exhibits enhanced deetiolation and senescence (Ling et al., 2012). Whether the TOC reorganization involving the ubiquitin-proteasome system accounts for proteomic changes

during the other plastid differentiation stages deserves future investigation.

Interestingly, genetic studies have suggested the involvement of SP1 in abiotic stress responses through proteolytic regulation of the protein-import apparatus (Ling and Jarvis, 2015). The Arabidopsis genome encodes two SP1 homologs, SP1-LIKE1/2 (SPL1/2), both of which are outer envelope proteins. SPL1 is the closest homolog of SP1, but its overexpression failed to complement the *sp1* mutation, likely due to distinct target specificity (Ling et al., 2012). Potential ligands for these E3 ligases include other outer envelope proteins such as enzymes involved in lipid metabolism, regulators for chloroplast division/movement, and an envelope-associated PHD transcription factor mediating retrograde signaling (Huang et al., 2013).

Quality Control of Protein Import by Clp

A portion of the ClpC chaperone present in chloroplasts is associated with the inner envelope membranes, where it is found in complexes with the protein translocation machinery (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). The N-terminal domain of ClpC is important for its envelope localization (Chu and Li, 2012). The ClpC protein associates with TIC110 but not with TIC40/55 (Flores-Pérez et al., 2016). Recently, it has been demonstrated that, along with ClpC chaperones, the ClpPR core also is attached to envelope membranes, whereas ClpD is localized exclusively in the stroma (Sjögren et al., 2014; Fig. 3). Through quantitative analysis of the ClpC-ClpPR stoichiometry, all envelope ClpC proteins (30% of total ClpC) are presumed to function together with the proteolytic core. Clp is unlikely to be involved in the maintenance of the TIC machinery itself (Sjögren et al., 2014). Rather, it seems to participate in a protein quality control mechanism for surveying preproteins being released from the TIC complex, in particular during transit peptide processing and subsequent refolding, to ensure the integrity of the chloroplast proteome (Sjögren et al., 2014). Indeed, the protein import efficiency is reduced in mutants for the proteolytic core as well as the chaperones. In addition, mutated Clp chaperone that is defective in protease core interaction fails to import proteins efficiently despite its normal envelope association (Flores-Pérez et al., 2016). Clp chaperones have the intrinsic ability to bind the N-terminal transit peptide as well as to renature nonnative proteins (Rosano et al., 2011; Bruch et al., 2012). ClpC interactions with the transit peptide and mature regions of preproteins being imported were detected recently (Huang et al., 2016). These observations suggest the involvement of the Clp system in clearing unprocessed or misfolded proteins generated during import. Alternatively, proteins being imported could aggregate and become stuck in the import channel. Such protein aggregates would have to be removed proteolytically for efficient protein translocation.

Comparative leaf proteomic studies of the Clp core mutants have shown a drastic loss of photosynthetic machineries that consist of numerous nucleus-encoded, imported proteins (for review, see Nishimura and van Wijk, 2015). Systematic downregulation of plastid-encoded proteins for photosynthesis but not for other functions such as translation, transcription, and fatty acid biosynthesis have been observed in the Clp core mutant. This fact is explainable by the accelerated degradation of plastid-encoded partner proteins that cannot assemble with nucleus-encoded photosynthetic proteins because of their inefficient import. Because a lack of functional photosynthetic complexes engenders severe phenotypes from chlorosis to albinism that resemble the Clp chaperone or core mutant phenotypes, the major function of plastid Clp, in terms of effects on growth and development, might be to eliminate aggregated, misfolded, or unprocessed preproteins of such photosynthesis machineries during protein import into the chloroplast (for a more extensive discussion, see Nishimura and van Wijk, 2015).

Case 2: Protein Degradation in Stroma-Facing Compartments (SPP, PrePs, OOP, and Clp)

Sequential Proteolytic Events for Preprotein Processing and Transit Peptide Removal

The aberrant accumulation of peptides generated by protein degradation or preprotein processing can affect plastid function and physiology and, therefore, must be removed. Upon arrival in the stroma, SPP binds with a broad spectrum of specificity to the transit peptides and removes them by a single endoproteolytic reaction, with subsequent release of the mature proteins but not the transit peptides (Richter and Lamppa, 1999, 2002). Recent proteome analysis showed that a substantial number of stromal proteins have multiple distinct N termini, implying the presence of multistep N-terminal processing mechanisms or simply reflecting SPP's imprecise site specificities (Rowland et al., 2015). The cleaved transit peptides are subjected to additional trimming into smaller fragments by SPP and are degraded further by PrePs and OOPs in the stroma (Teixeira and Glaser, 2013; Fig. 3). Transit peptides are 26 to 146 amino acids long (Zybailov et al., 2008). Structural and biochemical studies have reported that PreP1 degrades various peptide substrates of 10 to 65 amino acids (Moberg et al., 2003; Ståhl et al., 2005), whereas OOP cleaves peptide fragments ranging from eight to 23 amino acids (Kmiec et al., 2013). Consequently, both proteases are able to function in transit peptide degradation, with OOP acting in parallel or downstream to PreP. Furthermore, given the sizes of the degradation products generated by the proteases such as Clp and FtsH, PrePs and OOP can function downstream of these proteolytic machineries. However, whether and how the transit peptides are degraded

in vivo by PrePs and OOP (together with the other proteases) await further investigation.

Regulation of Metabolic Pathways

Chlorophyllide *a* oxygenase (CAO) is a key enzyme converting chlorophyll *a* to chlorophyll *b* during chlorophyll biogenesis. CAO protein stability is subject to negative feedback regulation in response to chlorophyll *b* metabolite levels (Yamasato et al., 2005). CAO degradation involves the Clp system (Nakagawara et al., 2007). CAO is localized in thylakoid and inner envelope membranes (Eggink et al., 2004), where it is accessible to Clp. A short degradation signal, the CAO degron, is located in the N-terminal domain of CAO (Sakuraba et al., 2007, 2009). A plausible model for metabolite-dependent CAO degradation has been proposed; in the model, the CAO degron would be located within the interior region in the absence of chlorophyll *b* but would be exposed to the exterior through a structural change in the presence of chlorophyll *b* such that the degron could be recognized by the protease (Sakuraba et al., 2009).

Glutamyl-tRNA reductase (GluTR) catalyzes the initial step generating Glu-1-semialdehyde from glutamyl-tRNA (Glu-tRNA) during tetrapyrrole biogenesis. GluTR is recognized directly through its N-terminal region by the ClpF-ClpS1 binary adaptor system as well as by ClpC chaperones for proteolysis (Nishimura et al., 2015; Apitz et al., 2016). GluTR is localized in the stroma and thylakoids. Its thylakoid localization is mediated in part by GluTR-binding protein (GBP; Czarnecki et al., 2011). GluTR abundance in the thylakoid is reduced in the absence of GBP, suggesting an accelerated destabilization of GluTR. A possible mechanism has been proposed in which Clp-dependent proteolysis and GBP-assisted stabilization together fine-tune GluTR accumulation to optimize chlorophyll and heme biosynthesis (Apitz et al., 2016). Glu-tRNA is a substrate not only for GluTR in the tetrapyrrole pathway but also for chloroplast ribosomes in translation; these two enzymes consume it competitively. The observation that the ClpS1-null mutant is sensitive to a translational inhibitor has led to the inference that ClpS1 can modulate chloroplast protein biosynthesis by regulating Glu-tRNA flux through GluTR degradation (Nishimura et al., 2013; Nishimura and van Wijk, 2015).

Degradation of a key metabolic enzyme, deoxyxylulose 5-phosphate synthase (DXS), in the methylerythritol 4-phosphate pathway for isoprenoid biosynthesis is regulated by metabolite levels (Guevara-García et al., 2005; Han et al., 2013). Furthermore, inactive DXS is likely recognized by a J protein, J20, which functions as an adaptor delivering misfolded or damaged client proteins to Hsp70 chaperone for either refolding or proteolysis depending on intracellular contexts (Pulido et al., 2013). The decision of dysfunctional DXS refolding versus degradation is proposed to involve the actions of stromal ClpB3 and ClpC1 chaperones; ClpB3 may function together with Hsp70 in DXS reactivation, while ClpC1 likely recognizes the enzyme for proteolysis (Pulido et al., 2016). Regulatory mechanisms of the nonfunctional protein fate decision involving multiple molecular chaperones deserve further investigation.

Several metabolic enzymes in the shikimate pathway of aromatic amino acid biosynthesis, including chorismate synthase, are potential targets for ClpS1-dependent proteolysis, perhaps suggesting the involvement of plastid Clp in the multistep regulation of various metabolic pathways (Nishimura et al., 2013; Nishimura and van Wijk, 2015). During plastid differentiation from etioplasts to chloroplasts in leaves, multiple enzymes in amino acid metabolism are decreased considerably in abundance, whereas Calvin cycle enzymes and thylakoid proteins accumulate to higher levels (Kleffmann et al., 2007). Possible involvement of the Clp system in this selective protein reduction awaits experimental verification.

Regulation of Metal Homeostasis

The copper transporter PAA2/HMA8 is an integral thylakoid membrane protein that delivers copper ions to plastocyanin (PC) in the lumen (Abdel-Ghany et al., 2005). PAA2/HMA8 abundance is down-regulated in response to high copper concentrations (Tapken et al., 2012). This copper-induced down-regulation involves proteolysis by the stromal Clp system rather than thylakoid-located FtsH protease (Tapken et al., 2015).

How the Clp system recognizes and degrades PAA2/HMA8 remains an important question. Genetic studies showing that its proteolytic regulation requires the ClpC chaperone and the ClpPR core but not the adaptor protein ClpS1 suggest that the chaperone directly recognizes the substrate depending on the copper level. PAA2/HMA8 has eight transmembrane domains, with the N-terminal domain, three internal loop structures, and the C-terminal tail exposed to the stromal side (Bernal et al., 2007). In particular, the N-terminal domain contains a metal-binding motif, which potentially induces a conformational change in response to elevated copper levels. This change might well trigger PAA2/HMA8 recognition by the Clp chaperone, similar to its CAO recognition. Another copper transporter, PAA1/HMA6, is present in the inner envelope membrane (Shikanai et al., 2003). Its accumulation is unaffected by high-copper conditions (Tapken et al., 2012). It is noteworthy that some of the ClpS1 targets isolated through affinity purification are metal-binding or metal-related proteins (Nishimura et al., 2013).

Case 3: Thylakoid Protein Biogenesis, Homeostasis, and Quality Control (Plsp1, Deg, and FtsH)

Regulation and Quality Control of Lumenal Targeting Peptide Removal

After import, most if not all lumenal proteins are sorted from stroma through thylakoid-localized SEC/TAT/SRP machineries to the thylakoid lumen. Protein

sorting requires TTS, which must be removed by TPP for protein assembly, posttranslocational release from the membrane, and, therefore, proper thylakoid formation (Inoue et al., 2005; Shipman-Roston et al., 2010; Midorikawa and Inoue, 2013).

Plsp1 is the TPP that functions in cleaving off TTS in the lumen (Shipman and Inoue, 2009). Knockout of Plsp1 causes the accumulation of processing intermediates of the SEC substrates, PC and OE33/PsbO, and the TAT substrate, OE23/PsbP (Shipman-Roston et al., 2010; Albiniak et al., 2012). The absence of Plsp1 and the elimination of TPP cleavage sites seem not to affect the SEC pathway itself but impede substrate release from the membrane; indeed, PC and PsbO intermediates are stuck in the thylakoid membrane despite their proper membrane targeting (Shackleton and Robinson, 1991; Frielingsdorf and Klösgen, 2007; Midorikawa and Inoue, 2013). It is noteworthy that precursor PsbO is stably present in a 440-kD complex that is not fully characterized but is redox sensitive, protease tolerant, and distinct from the PSII or SEC machinery, whereas unprocessed PC is prone to light-driven proteolysis by unknown protease(s). The PC degradation activity must be metal independent but is dependent on stromal proteins, the proton motive force across the thylakoid, and ATP hydrolysis. Thylakoid-retained PC cleavage intermediates are exposed in part to the stroma (Midorikawa and Inoue, 2013), potentially allowing the access of stromal proteases, as in the case of PAA2/HMA8 for Clp (Tapken et al., 2015). The PsbP intermediate is detected as a monomer in the stroma when Plsp1 is missing (Shipman-Roston et al., 2010; Midorikawa and Inoue, 2013) but exists within the membrane, presumably through an association with the TAT machinery, when its TPP processing site is abolished (Frielingsdorf and Klösgen, 2007; Midorikawa and Inoue, 2013). An earlier report has shown that TAT-dependent substrates can be returned to the stroma and that disruption of the TPP sites of the TAT substrates causes their unprocessed intermediates to accumulate in the thylakoids, where they are likely to be trapped in the sorting machinery (Di Cola and Robinson, 2005). A possible function of thylakoid Plsp1 in preventing reverse translocation has been proposed (Midorikawa and Inoue, 2013). Interestingly, a lumenal protein lacking the lumenal target peptide is prone to mislocalization in stroma, where it is readily degraded (Halperin and Adam, 1996). This observation represents an example of a lumenal protein quality control mechanism.

PSII Repair Cycle on the Thylakoid Membrane

Excess light exposure generates ROS, which can cause irreversible inactivation of photosynthetic machineries. The main target of photodamage is the D1 subunit of the PSII reaction center. D1 protein turns over very rapidly through a cyclical mechanism called the PSII repair cycle. Photodamaged PSII core complex migrates from grana to stromal thylakoids, where it is partially disassembled, enabling protease to access D1 for degradation. Following the insertion of de novo-synthesized D1, PSII is reassembled and migrates back into the grana (Chi et al., 2012; Nath et al., 2013; Yoshioka-Nishimura and Yamamoto, 2014).

FtsH and Deg proteases act in a cooperative manner in D1 degradation, in which lumenal and stromal Deg isomers function prior to FtsH (Kato et al., 2012; Fig. 3). D1 has five transmembrane-spanning helices (A–E). Deg5/8 and possibly Deg1 endoproteolytically cleave damaged D1 at a lumen-facing loop connecting the transmembrane helix domains C and D (CD loop), whereas Deg7 and Deg2 is likely to be responsible for the respective cleavage events occurring at stroma-exposed loops between transmembrane helices B and C and transmembrane helices D and E (Haussühl et al., 2001; Kapri-Pardes et al., 2007; Sun et al., 2007, 2010; Kato et al., 2012). D1 fragments resulting from cleavage at the lumenal CD loop are detected predominantly during high-light exposure (Kato et al., 2012), consistent with Deg activation through the oligomerization triggered by light-dependent lumenal acidification (Kley et al., 2011). D1 fragmentation by lumenal Deg can be initiated through disruption of the manganese cluster of the oxygen-evolving complex by blue light, fitting with the two-step model for photoinhibition (Kato et al., 2015), in which the manganese cluster in the oxygen-evolving complex is primarily damaged and the PSII reaction center is then inactivated by chlorophyll-absorbed light energy (Hakala et al., 2005; Ohnishi et al., 2005). Whether Deg functions before, during, or after PSII migration remains unknown. Deg-generated D1 fragments are degraded further through FtsH-dependent processive proteolysis. The four major isoforms FtsH1/2/5/8 together function in this process, but whether FtsH6 is involved is not known. FtsH can initiate D1 degradation even in the absence of the Deg proteases, possibly by pulling the stroma-oriented N-terminal tail of the D1 subunit, but the degradation efficiency is enhanced in the presence of Deg proteases (Kato et al., 2012). In cyanobacteria, the D1 N-terminal tail is exposed outside the PSII complex. The absence of this N-terminal tail inhibits D1 degradation, likely because of failure in N-terminal recognition by FtsH (Komenda et al., 2007).

The repair cycle of photodamaged PSII is modulated through its reversible phosphorylation. The PSII core proteins D1, D2, CP43, and PsbH can be phosphorylated; the phosphorylation sites are all located in their stroma-facing N termini (Puthiyaveetil and Kirchhoff, 2013). The phosphorylation and dephosphorylation of these proteins require the thylakoid-associated Ser/Thr kinase STATE TRANSITION8 (STN8; Bonardi et al., 2005) and the stroma/thylakoid-localized PROTEIN PHOSPHATASE 2C-TYPE PSII CORE PHOSPHATASE (PBCP; Samol et al., 2012). Lack of STN8 results in enhanced Deg-dependent D1 fragmentation under high-light conditions (Kato and Sakamoto, 2014), whereas PBCP defects cause delayed D1 degradation in high light (Samol et al., 2012). Levels of smaller,

intermediary D1 fragments are correlated with ROS accumulation, suggestive of their cytotoxicity. These observations have led to the idea that PSII phosphorylation prevents excessive D1 degradation to avoid the accumulation of cytotoxic cleavage intermediates. Furthermore, given that phosphorylation of PSII core proteins can promote PSII migration and disassembly through structural remodeling of the thylakoid membrane to facilitate D1 access to the proteases (Kirchhoff, 2013), it is plausible that PSII phosphorylation fine-tunes the repair cycle by balancing D1 presentation and degradation (Kato and Sakamoto, 2014). How PSII phosphorylation modulates D1 proteolysis requires further investigation.

Proteolytic Remodeling of the Photosynthetic Machineries in Response to Environmental Stimuli

In the unicellular alga *Chlamydomonas reinhardtii*, FtsH participates together with Clp and unidentified lumenal proteases in the selective degradation of the cytochrome b_6/f complex and its biogenesis factors upon nitrogen starvation (Wei et al., 2014). Their mechanisms of action in cytochrome b_6/f degradation, however, are apparently different, because destabilization of the cytochrome b_6/f complex triggered by biogenesis and assembly problems is blocked in the absence of FtsH but not in the absence of Clp (Majeran et al., 2000; Malnoë et al., 2014). The nitrogen-depletion-stimulated cytochrome b_6/f degradation is presumably controlled by a signaling pathway involving nitric oxide (Wei et al., 2014). FtsH-dependent cytochrome b_6/f degradation is found in sulfur-starving algal cells as well (Malnoë et al., 2014). In addition, algal FtsH participates in PSII destruction during phosphorus-starvation and sulfur-starvation conditions, presumably through a proteolytic mechanism similar to the repair cycle (Malnoë et al., 2014).

The aforementioned macronutrient-responsive proteolytic regulation mechanisms involving algal FtsH and Clps might well be conserved in land plant chloroplasts. Furthermore, the observations that a lack of stromal Clp induces the up-regulation of thylakoid-localized FtsH and SppA proteases (Rudella et al., 2006) and that the loss of FtsH engenders the up-regulation of other chloroplast proteases, including Clp and SppA, and the recruitment of stromal Clp to the thylakoid (Kato et al., 2012) suggest that they play mutual compensatory roles, thereby implying conservation of their coordinated actions in the remodeling of the photosynthetic apparatus upon stress.

FUTURE PERSPECTIVES

Complete Sets of Substrates, Degron, and Substrate Recognition Mechanisms?

Specific degradation signals, namely degrons, are necessary for chaperones and/or adaptor proteins of the proteolytic systems to recognize protein substrates for destruction (e.g. ClpS), but such degron sequences are poorly understood in plastids (see Outstanding Questions). A recent study has identified a novel N-terminal degron for FtsH-driven proteolysis in bacteria (Bittner et al., 2015), raising the question of whether such a target recognition mechanism is conserved in chloroplasts. Meanwhile there have been several well-known examples of proteolytic regulation of specific chloroplast proteins. Specifically, light-harvesting antenna size has long been known to be strictly regulated in response to elevated light intensities, and apoproteins of LHCII are thought to be proteolytically degraded (Jansson, 1994). However, the responsible protease is unknown. Comprehensive substrate identification is necessary for determination of a common sequence signature or regulatory motif. Using substrate-trapping approaches, multiple substrates for Clp and FtsH proteases have been isolated in bacteria and in mammalian mitochondria (Flynn et al., 2003; Neher et al., 2003; Westphal et al., 2012; Bhat et al., 2013; Feng et al., 2013; Graham et al., 2013; Bittner et al., 2015). Trapping strategies use mutated, inactive forms of the protease or chaperone domains. By substituting catalytically active with catalytically inactive degradation machineries fused to a specific tag, the in vivo substrates are confined within and purified in complexes with protease assemblies from the cell. Similar methodologies are worth pursuing for identification of substrates for chloroplast proteases.

Regulatory Circuits Involving Multiple Proteases to Optimize Interorganellar Proteostasis

Chloroplast proteases can function in a compensatory manner (see "Outstanding Questions"). FtsH protease has long been known to interact genetically with the Clp system (Park and Rodermel, 2004; Yu et al., 2008; Wu et al., 2013). Knowledge of this interaction is based on the observation that the loss of Clp chaperone or protease core suppresses the leaf variegation phenotype resulting from the lack of FtsH2, a major component of the FtsH machinery. It is particularly interesting that ClpC2 is not the major ClpC chaperone but its reduction is somehow sufficient for the suppression of a variegated leaf phenotype resulting from FtsH2 loss of function, despite the fact that ClpC1 is abundantly present in the suppressor mutant (Park and Rodermel, 2004). More importantly, the Clp chaperone and proteolytic core are both up-regulated and recruited to the thylakoid membrane in the mutant lacking FtsH2, suggesting their complementary roles for FtsH deficiency (Kato et al., 2012). FtsH isomers are up-regulated in the Clp core mutant, consistent with their functional compensation (Rudella et al., 2006). Similar responses to the Clp core defect have been observed for PrePs (Zybailov et al., 2008). These findings suggest that the proteases share a common substrate recognition mechanism or that they can recognize identical substrates in a distinct manner. Further experimentation is needed to examine these proposed compensatory mechanisms.

OUTSTANDING QUESTIONS

- Despite recent elucidation of substrate proteins for the major chloroplast proteases, we still need to develop a systematic approach for identifying in vivo targets to fully understand substrate recognition mechanisms.
- Mechanistic insights into functional interactions among multiple plastid proteases require further investigation.
- A possible regulatory mechanism of interorganellar proteome homeostasis involving dual-targeted proteases deserves particular attention and future evaluation.

Furthermore, dual targeting proteases could potentially regulate proteome homeostasis in both organelles (see "Outstanding Questions"). FtsH11, Lon4, PrePs, and OOP are targeted to both mitochondria and chloroplasts (Moberg et al., 2003; Kmiec et al., 2013). Given that the distribution of a dual-targeted protein is altered by organellar stress and dysfunction (Nargund et al., 2012), allocation of these protease and peptidase machineries between the chloroplast and mitochondrion could be regulated at the protein import step, depending on the status of organellar protein homeostasis. For the coordinated biogenesis and the maximum function of two organelles, reprogramming of the nuclear transcriptome by proteases in both organelles might contribute to the optimization of interorganellar proteomes.

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