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Review

Organization, function and substrates of the essential Clp protease system in plastids☆

Kenji Nishimura, Klaas J. van Wijk *

Department of Plant Biology, Cornell University, Ithaca, New York 14853

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ABSTRACT

Intra-plastid proteolysis is essential in plastid biogenesis, differentiation and plastid protein homeostasis (proteostasis). We provide a comprehensive review of the Clp protease system present in all plastid types and we draw lessons from structural and functional information of bacterial Clp systems. The Clp system plays a central role in plastid development and function, through selective removal of miss-folded, aggregated, or otherwise unwanted proteins. The Clp system consists of a tetradecameric proteolytic core with catalytically active ClpP and inactive ClpR subunits, hexameric ATP-dependent chaperones (ClpC,D) and adaptor protein(s) (ClpS1) enhancing delivery of subsets of substrates. Many structural and functional features of the plastid Clp system are now understood though extensive reverse genetics analysis combined with biochemical analysis, as well as large scale quantitative proteomics for loss-of-function mutants of Clp core, chaperone and ClpS1 subunits. Evolutionary diversification of Clp system across non-photosynthetic and photosynthetic prokaryotes and organelles is illustrated. Multiple substrates have been suggested based on their direct interaction with the ClpS1 adaptor or screening of different loss-of-function protease mutants. The main challenge is now to determine degradation signals (degrons) in Clp substrates and substrate delivery mechanisms, as well as functional interactions of Clp with other plastid proteases. This article is part of a Special Issue entitled: Chloroplast Biogenesis.

1. Plastid proteases and proteolysis in biogenesis, differentiation and proteostasis

The dynamic plastid proteome Higher plant plastids undergo major changes in morphology, membrane and proteome composition and function during developmental transitions (*e.g.* embryogenesis, leaf development, senescence, fruit ripening), and more subtle changes in response to abiotic stress. Such plastid proteome dynamics requires regulated proteolysis together with transcriptional and translational regulation. Indeed, distinct proteome landscapes have been shown for different types of plastids [1–6] and substantial changes in proteome composition during chloroplast development, including proplastid-to-chloroplast, etioplast-to-chloroplast and chloroplast-to-chromoplast transitions in various plant species [7–9]. These studies underscore the notion that protein degradation is a key process in plastid homeostasis and differentiation, and that this must involve careful selection of protein substrates.

Plastid proteases are needed for protein maturation Proteases are needed in the preprotein processing and maturation of plastid proteins. About 100 proteins are synthesized in plastids and in most cases their N-terminal methionine residue is co- or post-translationally removed by methionine amino-peptidases, presumably to improve protein functionality and/or protein stability. However, most plastid proteins are encoded in the nuclear genome and synthesized in the cytosol as precursors with N-terminal cleavable targeting peptides (cTPs), followed by import into plastids across the envelope membranes through the TOC/TIC translocation complex [10]. Imported pre-proteins are subject to cTP cleavage by the general stromal processing peptidase (SPP) [11,12] and possibly additional N- or C-terminal processing steps [13]. The cleaved cTPs represent a significant amount of protein mass but are rapidly degraded, perhaps by the Prep and OOP proteases [13–16]. However, this recycling process has not been well characterized and may involve additional proteases.

Proteolysis is needed for removal of dysfunctional or unwanted proteins Proteolysis is also required for general plastid proteome homeostasis. For example, the light-harvesting antenna size is regulated by the balance of protein synthesis and degradation in response to changing light intensities. Other examples include the developmental regulation of the essential plastid methyl-D-erythritol 4-phosphate (MEP) pathway generating isoprenoids [17,18]. Many plastid proteins are present as hetero-oligomeric complexes, and unassembled proteins are typically rapidly removed by proteolysis, as demonstrated in numerous studies in higher plants and the green algae *Chlamydomonas reinhardtii* [19,20]. The D1 protein of Photosystem II (PSII) has a relatively short life-time and damaged D1 protein is efficiently removed by proteolysis

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* Corresponding author at: Dept. of Plant Biology, Emerson Hall 332, Cornell University, Ithaca, NY 14853. Tel./fax: +1 607 255 3664.
E-mail address: kv35@cornell.edu (K.J. van Wijk).

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involving FtsH and Deg proteases and perhaps the Clp proteases - reviewed in [21]. If plastid proteins are improperly folded or become partially unfolded during their life-time (*e.g.* by elevated temperatures) this can result in their inactivation or aggregation; these miss-folded or aggregated proteins likely can become toxic for the plastid and must then be removed by proteolysis. It is not clear which plastid proteases remove such dysfunctional proteins, but the Clp protease system is likely involved.

The Clp protease system in plastids Multiple proteases have been discovered in chloroplasts through biochemical, genetics, bioinformatics or proteomics approaches. The physiological significance of several of these proteases in plastid biogenesis, embryogenesis and plant development has been demonstrated [13,22]. The ATP-dependent serine-type Clp protease system is the most abundant stromal protease in (developing) chloroplasts. The first plastid-localized Clp component was discovered in 1993 by Keegstra in spinach as a protein predominantly localized in the stroma but also present in the chloroplast envelope [23]. This was quickly followed by the discovery of chloroplast ClpP1 and even demonstrating that recombinant ClpC could aid the *E. coli* Clp protease in degradation of a model substrate [24]. In 2001, it was realized that the chloroplast Clp protease core complex has a surprisingly complexity, in particular when compared to Clp proteases in prokaryotes [25]. Moreover, it was shown that the plastid-encoded ClpP1 gene is essential [26]. The sequencing of the Arabidopsis genome, the emergence of the Arabidopsis T-DNA insertion collection and rapid improvement of protein mass spectrometry have since facilitated extensive analysis of the physiological significance, as well as organization and assembly state of the Clp system in higher plant plastids. This research was last extensively reviewed at the end of 2010 [27], but since then more than a dozen new experimental papers about the Clp system in Arabidopsis were published, and direct evidence for Clp substrates has been obtained. Furthermore, the significance of the Cp system is now also demonstrated for the important monocotyledon crop species maize and rice. Here we provide an up-to date comprehensive review about the organization and functions of the plastid Clp protease system, in which also major questions and new research directions are discussed. Comparison of Clp protease system in plastids, cyanobacteria and non-photosynthetic organism highlights unique features and diversification of the plastid Clp system and hypotheses for substrate recognition and delivery will be presented.

2. General functional organization of the Clp system

The basic structure of the Clp machinery is generally conserved throughout evolution and consists of a cylinder-like or barrel-like protease core and an AAA^+ (ATPase Associated with various cellular Activities) chaperone ring complex (Fig. 1A). The chaperone complex serves as a molecular gate that controls substrate access, and recognizes, unfolds and translocates protein substrates into the core cavity in an ATP-dependent manner. High resolution structural information has been obtained from NMR and X-ray crystallography for non-photosynthetic bacterial Clp components, including the Clp core, the chaperones and ClpS domains in complex with chaperones (reviewed in [28]). So far no high resolution structures have been determined for Clp components in photosynthetic organisms; however it is most likely that many structural aspects are conserved. Based on structural data from *E. coli*, we generated a homology model for the Arabidopsis Clp core and chaperone complex (Fig. 1B) and see [29]. Structural informational for bacterial Clp core complexes has shown that the flexible entrance pore of ~10-17 Å is too narrow for native proteins to enter the proteolytic chamber [30]. The catalytic residues of the protease are facing into the core cavity; this sequestering of the proteolytic activity avoids unspecific degradation of proteins. In bacteria it has been firmly established that substrates are processively

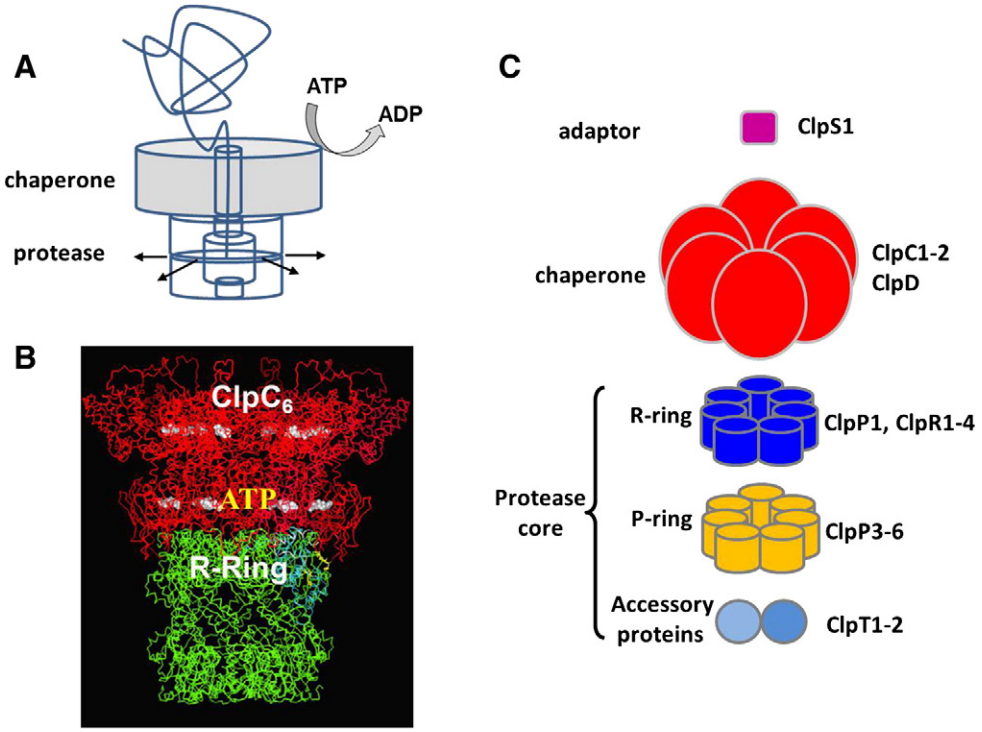

Fig. 1. Clp organization in Arabidopsis plastids. **A**. General overview of Clp structure. The Clp chaperone unfolds and translocates protein substrates into the proteolytic core in an ATP-dependent manner. The substrates are degraded into small peptides that are emitted through the lateral pores of the core chamber. **B**. Homology model of the Arabidopsis ClpPR core assembled with the ClpC chaperone ring. ClpC hexamer and ClpPR tetradecamer are indicated in red and green, respectively. ATP molecules are also shown in white. This model was generated by D. Ripoll (previously unpublished) as part of our analysis of the organization of the ClpPR core [29]. **C.** Plastid Clp system consists of 1) ClpS1 (purple) as the adaptor protein, 2) ClpC1/C2/D (hexamer, red) as the chaperone subunits, 3) ClpP1/R1-4 (heptamer, marine blue) for R-ring, 4) ClpP3-P6 (heptamer, orange) for P-ring, 5) ClpT1 (pale blue) and ClpT2 (blue) as the accessory proteins. Two asymmetric rings together with two accessary proteins constitute the proteolytic core.

degraded into smaller peptide fragments of 5–10 amino acids in length inside the core cavity [31,32], which are then ejected though dynamic narrow lateral pores. Homology modeling already suggested such lateral pores in the chloroplast Clp protease core prior to these reports for bacterial Clp [29]. In bacteria, it has been shown that the protease core alone displays slow degradation activity against small peptides (less than 6 amino acids in length) but it requires the chaperone component for proteolysis of longer peptides or proteins. Consistently, ATP hydrolysis is needed for degradation of proteins but not of peptides [33]. The Clp protease shows several organizational similarities to the 26S proteasome [34].

3. Evolution and the diversity of Clp components

For discovery, functional and structural understanding of the Clp protease system in photosynthetic organisms, it is very helpful to consider information for Clp protease systems along the evolutionary 'tree'. Fig. 2A summarizes the Clp components and organization in the five major subgroups of bacteria (proteobacteria, firmicutes, spirochates, actinobacteria, cyanobacteria) and in various plastid types (apicoplast, non-photosynthetic plastid, chloroplast) and mitochondria in eukaryotes (Fig. 2A). There are two major types of Clp core complexes, namely the tetradecameric serine protease formed by two stacked heptameric rings of ClpP proteases (and/or related non-catalytic ClpR proteins; see below) [35,36] (Fig. 2A) and dodecameric Clp complexes formed by two stacked hexameric rings of ClpQ proteins (also named HslV) (not shown). The AAA^+ chaperone subunits are categorized into class I and II, respectively containing double or single AAA domains. Class I includes ClpA in proteobacteria, actinobacteria and spirochaetes, ClpC in firmicutes and actinobacteria, and ClpE in firmicutes. Class II contains ClpX in nearly all bacterial species and ClpY (HslU) in proteobacteria, firmicutes, spirochaetes, aquificae and thermatogae (reviewed in [28]). ClpA/ClpC/ClpE/ClpX chaperones form homohexamers that can bind to the ClpP core, while ClpY hexamers interact exclusively with the ClpQ core. Substrate recognition abilities of the Clp chaperones are modulated by their cognate adaptor proteins, namely i) ClpS for ClpAP and ClpCP, ii) MecA, YpbH and McsB for ClpCP, iii) RssB, SspB, UmuD and YjbH for ClpXP. So far no adaptors have been found for ClpEP or ClpYQ - reviewed in [37]. Most bacterial species bear a single *clpP* gene but some firmicutes, spirochaetes, proteobacterial and actinobacterial species harbor multiple copies of *clpP* genes. The genes for chaperones (ClpA/ClpC/ClpE/ClpX) and adaptors (ClpS, MecA, SspB and UmuD), but not *clpQ* and *clpY*, are also duplicated in some bacteria.

Moving up the evolutionary ladder, in cyanobacteria, the *clpP* gene is also duplicated but further diversified to generate another component, ClpR, which is a proteolytically-inactive subunit of the protease core due to the loss of the catalytic triad [38]. The cyanobacterial Clp system is comprised of a hetero-tetradecameric ClpP1P2 core and a heterotetradecameric ClpP3R core (Fig. 2A). Furthermore, ClpX is the chaperone for the ClpP1P2 core, whereas ClpC is the chaperone for ClpP3R core. ClpS1 and ClpS2 are adaptor proteins interacting with the ClpCPR core [39]. Most cyanobacteria contain an additional component, NblA, which is a specific adaptor protein to deliver phycobilosome proteins to the ClpCPR system [40]. *nblA* is usually a single gene but some cyanobacterial strains harbor two such genes [41].

The Clp protease in higher plants originates from its ancient cyanobacterial endosymbiont, but it increased in complexity since endosymbiosis. Indeed, higher plant plastids and chloroplasts contain the most diversified Clp protease core compared to all other species. The plastid Clp system in *Arabidopsis thaliana*, consists of five ClpP

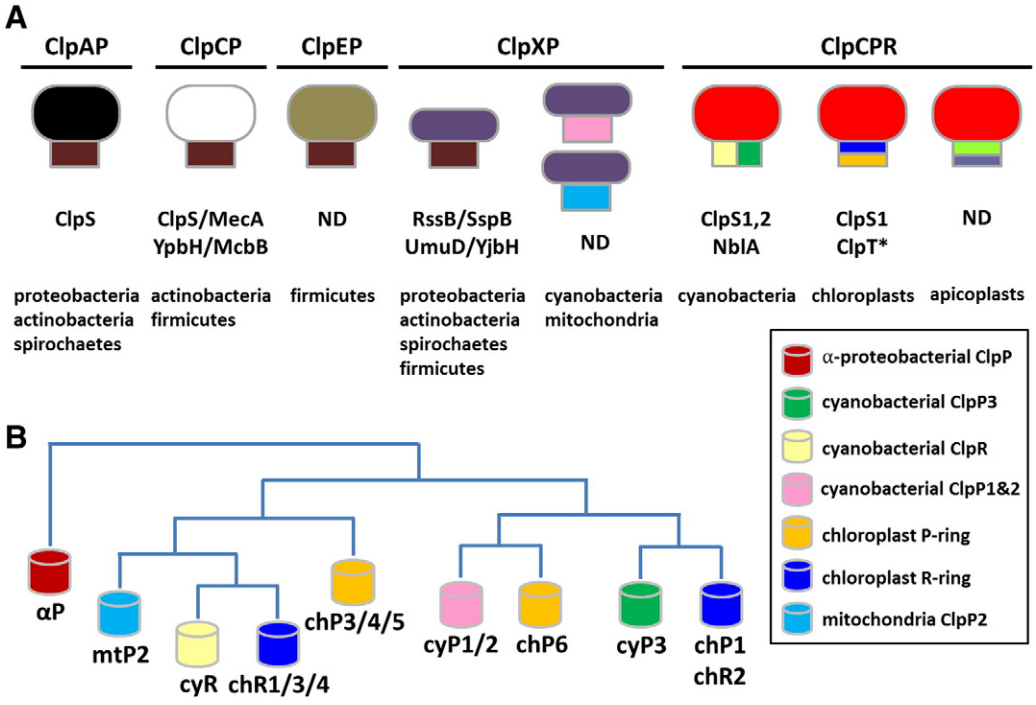

Fig. 2. Clp evolution and diversity. **A.** Diversity of Clp assemblies. Bacterial and organellar Clp systems are classified into nine types based on the structures and composition of AAA^+ chaperone and core assemblies. Class I chaperones (ClpA, ClpC, ClpD, ClpE) contain double AAA domains and Type II (ClpX and ClpY) contain a single AA domain. ClpA (black) is found in proteobacteria (*e.g. Escherichia coli*), actinobacteria (*e.g. Streptomyces coelicolor*) and spirochaetes (*e.g. Leptospira interrogans*). ClpC (white) exists in actinobacteria (*e.g. Mycobacterium tuberculosis*) and firmicutes (*e.g. Bacillus subtilis*). ClpE (olive) resides in firmicutes (*e.g. Streptococcus pneumoniae*). ClpX (purple) is widely distributed in most bacteria and eukaryotic mitochondria. ClpC (red) is present in cyanobacteria, eukaryotic chloroplasts and parasite apicoplasts. Clp cores are color-coded according to Fig. 2B right panel except bacterial ClpP (wine red), apicoplast R-ring (light green) and P-ring (indigo). Clp chaperones and cores are shown as ellipses and rectangles, respectively. Cyanobacterial Clp core contains two symmetric ClpP3/R heptamers. Chloroplast and apicoplast Clp cores consist of two asymmetric rings; each ring is heteromeric in chloroplasts but homomeric in apicoplasts. The adaptor proteins are indicated below if identified. ND, not determined. **B.** Evolutionary lineage of Clp core subunits. The simplified phylogeny (left panel) is based on [43]. Each branch length is arbitrary. The color-code of the Clp core components is shown in right panel; αproteobacterial ClpP (Dark red), cyanobacterial ClpP3 (green), cyanobacterial ClpR (pale yellow), cyanobacterial ClpP1/2 (pink), chloroplast P-ring subunits (orange), chloroplast R-ring components (marine blue) and mitochondria ClpR2 (light blue).

subunits (ClpP1, ClpP3, ClpP4, ClpP5 and ClpP6) and four ClpR subunits (ClpR1 to ClpR4) that form a single 325–350 kDa tetradecameric protease core, two ClpC (ClpC1 and ClpC2) and one ClpD chaperone, adaptor ClpS1, and two plant-specific accessory proteins, ClpT1 and ClpT2 (Fig. 1C). ClpP1 is the only plastid-encoded Clp subunit [27]. The 325–350 kDa plastid ClpPR core is constructed from the heptameric P-ring (~177 kDa) containing the four ClpP subunits (ClpP3 to ClpP6), and the heptameric R-ring (~189 kDa) containing the four ClpR proteins and ClpP1 [29,42,43]. This diversification of the plant Clp system already began early in the green lineage [43]. Phylogenetic analysis suggests that subunits of the R ring originate from the cyanobacterial ClpPR components; plant ClpP1 and ClpR2 derived from an ancestor of the cyanobacterial ClpP3, while ClpR1, ClpR3 and ClpR4 were generated from duplications of the ClpR in ancient cyanobacteria [43]. Whereas ClpP1 is a plastid-encoded subunit, ClpR2 subunit is encoded in the nucleus genome, possibly due to lateral gene transfer of the ancient cyanobacterial *CLPP3* to the nucleus followed by the intensive sequence modifications. The origins of the P-ring subunits (P3,4,5,6) are not clear, although ClpP6 is evolutionarily related to ClpP2 in cyanobacteria. The ClpT1,2 subunits are proteins of ~20 kDa with high homology to the N-terminal domain of ClpC chaperones. One or more ClpT genes are found in all sequenced plant species, and distantly related ClpT proteins (assigned ClpT3 and ClpT4) are found in Chlamydomonas chloroplasts [44,45]. ClpT proteins are absent in prokaryotes and apicomplexa. The presence of ClpT proteins does not coincide with the presence of ClpR proteins, nor are they correlated with the presence of a hetero-oligomeric Clp protease core. Thus, ClpT appears to be an evolutionary 'invention' in photosynthetic eukaryotes (Fig. 2A); possible functions of ClpT will be discussed in the next section. Since ClpT1 and ClpT2 proteins are missing in cyanobacteria but they have high sequence homology to the N-terminal domain of the ClpC proteins, the two ClpT paralogs are presumably generated through duplication in higher plants progenitors of the ClpC chaperone gene in ancestral cyanobacteria. ClpT3 and ClpT4 in the green algae also contain N-terminal part of the Clp chaperone, but phylogenetic analysis shows that they are not included in the same clade as land plant ClpT1/T2 proteins [45]. Compared to ClpT4, ClpT3 has further diversified to obtain an additional C-terminal extension with no homology to other known proteins. Thus ClpT1/T2 and the ClpT3/T4 proteins likely represent different gene duplication events.

Chloroplasts of higher plants and green algae contain ClpC and ClpD AAA+ chaperones. The only adaptor proteins so far found in higher plant plastids are ClpS1 homologs [46]. ClpS1 was detected by mass spectrometry analysis of the chloroplast stromal proteome of Arabidopsis [47]. We designated this homolog as ClpS1, since it is evolutionarily related to cyanobacterial ClpS1, but not ClpS2 [46]. Thus cyanobacterial ClpS1 evolved through the green lineage from algae to higher plants, while ClpS2 was evolutionarily 'left behind' and only found in photosynthetic prokaryotes. Whereas ClpX proteins are present in cyanobacteria, they are not present in plastids. Instead ClpX chaperones are found in plant mitochondria together with a homotetradecameric ClpP2 protease core [27]. Finally, apicomplexa are parasitic protists that contain a type of plastid called the apicoplast [48]. The malaria-causing *Plasmodium falciparum* is a highly studied member of the apicomplexa and its apicoplasts contains both a ClpP and ClpR protein but they appear not to form heteromeric complexes [49]. A single ClpC chaperone likely serves the Clp protease in this apicoplast [50].

4. Organization, assembly and stability of the chloroplast Clp protease core in Arabidopsis and Chlamydomonas

The native Clp protease core composition and organization in higher plant plastids has been studied in fair detail for chloroplasts in *Arabidopsis thaliana*. Multiple ClpP,R,T are present in maize, rice and other species, but the Clp core has not been purified and analyzed in monocots. The Clp core has also been studied in detail in the green algae Chlamydomonas. In this section we summarize current understanding of the composition, stability and assembly of the ClpPR core in Arabidopsis and Chlamydomonas. These nine different ClpPR proteins also form ClpPR core complexes in non-photosynthetic plastids of roots and leucoplasts in petals of respectively *Brassica rapa* and *Brassica oleracea* [29]. ClpPR proteins are found in proplastids in maize leaves and they are present through all developmental stages of developing leaf bundle sheath and mesophyll cell specific chloroplasts, but with the highest accumulation levels in early stages of chloroplast development [51]. The Clp system is also detected in pea etioplasts as one of the most prominent soluble complexes [52] and several Clp proteins were also found in chromoplasts [8]. Thus, it seems that the plastid Clp protease system is present in all plant plastid types as a constitutive macromolecular enzyme with total accumulation levels influenced by development and organ.

Using *in vivo* affinity tagging and purification of individual ClpP and ClpR rings, as well as the complete ClpPRT complex, using StrepII-tagged ClpR4 and StrepII-tagged ClpP3 followed by absolute quantification (QConCat) by mass spectrometry, it was determined that the stoichiometry for the P ring is ClpP3:P4:P5:P6 = 1:2:3:1 whereas the ratio for the R ring is ClpP1:R1:R2:R3:R4 = 3:1:1:1:1 [43] (Fig. 3). Thus the P-ring contains seven catalytic sites, and the R-ring contains only three catalytic sites. The R ring in plastids has a 3:4 ClpP:ClpR ratio, which is reminiscent of active/inactive subunit ratios of the cyanobacterial ClpP3/R core and the eukaryotic 20S proteasome [43]. Such similar 3:4 active:inactive subunit ratio may reflect a structural/functional limitation on the stoichiometry of these proteolytic machineries. It is not known what happens to ClpPR core activity in plastids if the number of active sites in the P-ring would be reduced, but experiments are in progress to test this (Liao, Kim and van Wijk, unpublished).

Primary sequence comparison shows that ClpP subunits share sequence identities between 24 to 48%, whereas ClpR proteins have 28 to 38% identities to each other [89], suggesting both structural and functional divergence even within the ClpP and ClpR subfamilies. However, which ClpP,R subunits interact with each other within each ring is unknown and extensive homology modeling did not suggest any preferential orientation within the ClpPR rings [29].

ClpT proteins have been proposed to be essential in facilitating the association between the P-ring and R-ring [53], in part because no double mutants for ClpT1 and ClpT2 could be obtained by the authors. In contrast, we recently obtained viable double mutants (Kim et al, in preparation). The reason that no double mutants were recovered in [53] is likely that *CLPT1* and *CLP2* are located closely together on the same chromosome resulting in non-Mendelian segregation rates in the progeny of such crosses. It was suggested that first ClpT1 attaches to the P-ring to form a stable complex, which then binds to ClpT2. Subsequently this ClpP-ring-T1,T2 complex then associates with the R ring to form the tetradecameric protease core. Addition of recombinant ClpT1,2 proteins to wild-type stroma increased the amount of ClpPR core on native gels, suggesting that availability of ClpTs is the rate-limiting factor for the core assembly. It was further speculated that ClpT1,2 directed assembly allows regulation of the ClpPR proteolytic in response to need to intraplastid proteolysis. In this model most ClpP and ClpR rings reside as individual rings in the stroma, and only upon an increased need, they assemble with the help of ClpT1,2 [54]. However, in our opinion this model is highly speculative and lacks experimental support. It was recently reported that recombinant ClpT1 can interact with recombinant ClpC2 in vitro [55], but we could not confirm this interaction (Nishimura and van Wijk, unpublished). Claims that ClpT1 regulates ClpC2 will require more specific experiments with appropriate negative controls.

Nuclear-encoded catalytically active ClpP (ClpP3 to ClpP6) and catalytically inactive ClpR (ClpR1 to ClpR4) subunits have plant-specific extended C-termini (up to 52 aa in length) which are not proteolytically cleaved during the core assembly [43]. These C-terminal extensions are predicted to fold over the top of the core structure, potentially affecting

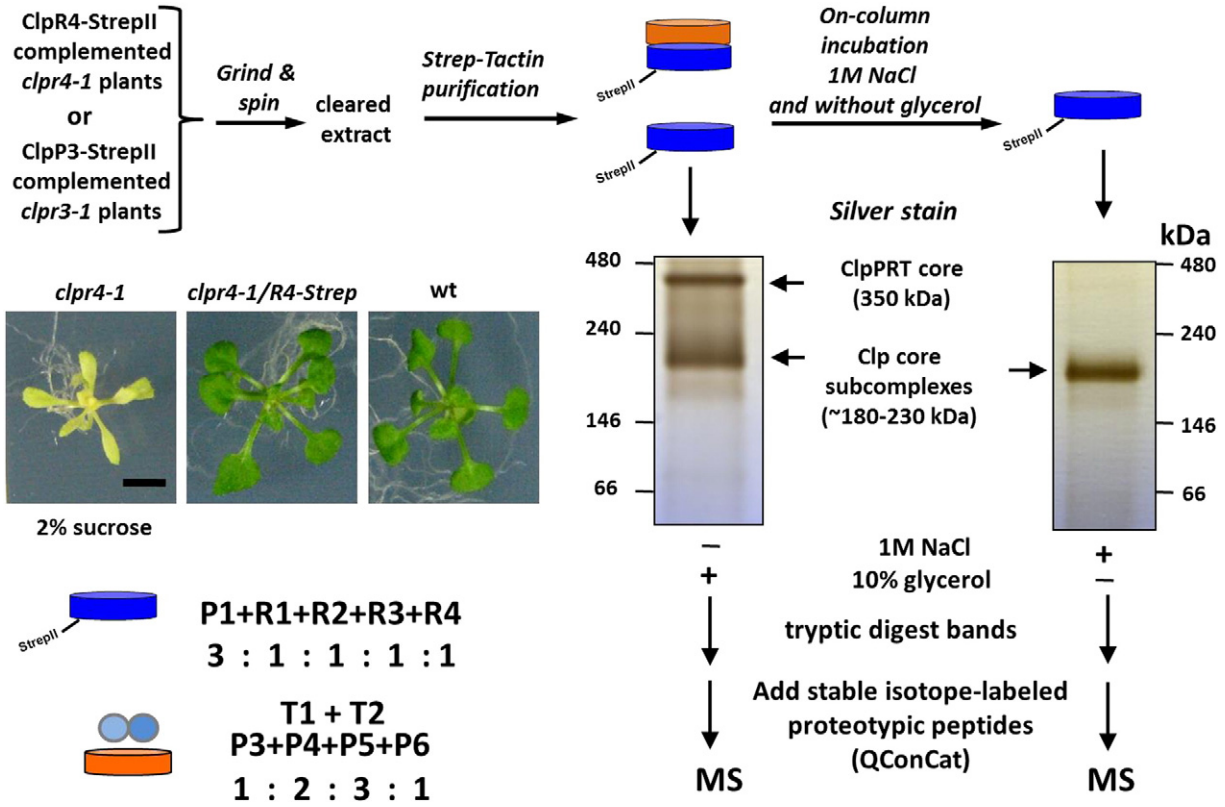

Fig. 3. Determination of the absolute stoichiometry of the subunits in the ClpP and ClpR ring by in vivo StrepII tagging, affinity purification and mass spectrometry-based quantification using spike-in stable isotope labelled peptides. Schematic illustrates the procedure for the absolute quantification of plastid Clp core composition. ClpPRT core is isolated from soluble lysates of clp mutant plants complemented with StrepII-tagged ClpR subunits through Strep-Tactin purification strategy. Clp core is stable in the presence of glycerol while on-column salt incubation in the absence of glycerol allows the core to dissociate into the subcomplexes (ie. ClpP- or ClpR-rings). Purified Clp intact core or subcomplex rings are separeted on the native gel followed by MS/MS analysis with stable isotope-labeled proteotypic peptides (QConCat). Images of plants grown on MS agar plates containing 2% sucrose demonstrate that StrepII-tagged ClpR4 subunit functionally complement the clpr4-1 phenotype. Representative gels stained with silver nitrate are shown. Quantified stoichiometry of each ring is indicated.

the interaction with the chaperone [29]. Several of the ClpPRT subunits have smaller observed masses that predicted after removal of the cTPs; this must be due to additional N-terminal processing of Clp subunits, following cleavage of the cTPs, and might be part of a mechanism for ClpPR core assembly. In *E. coli*, the N-terminus of ClpP is autocatalytically cleaved but it is not clear how this relates to the (self)-assembly process.

A short (9–10 aa) insertion sequence (named L1 insertion) is found in ClpR1, ClpR3 and ClpR4, but not in ClpR2 and ClpP subunits, and was proposed to influence substrate entry in the catalytic cavity based on homolog modeling [29]. The cyanobacterial ClpCPR shows a significantly lower degradation rate of a model substrate in comparison with ClpAP in *Escherichia coli* [56]. Introducing the catalytic triad into the ClpR protein, along with removal of the short insertion sequence, destabilized the core complex [56]. Perhaps these observations are related to the similarity in fixed active:inactive subunit stoichiometry among the various proteolytic assemblies (*i.e.* plastid R ring, cyanobacterial ClpP3/R and eukaryotic 20S) see [43].

Chloroplasts of the green algae *Chlamydomonas reinhardtii* also have a ClpPR protease core consisting of three ClpP (ClpP1,4,5) and five ClpR (ClpR1,2,3,4,6) proteins [44]. Similar to higher plants, Chlamydomonas ClpP1 and ClpR1-ClpR4 form the R-ring, while the nuclear-encoded ClpP subunits form the P-ring [45]. The Chlamydomonas ClpPR core size is ≈ 540 kDa, which is much higher in mass than plastid core in higher plants (325–350 kDa). There are four variants of the chloroplast-encoded ClpP1 subunit, one large form $ClpP1_H$ (52 kDa) and three smaller variants $ClpP1_N$, $ClpP1_C$ and $ClpP1_{C'}$ (between 22 and 24 kDa), all of which are part of the proteolytic core [45,57]. $ClpP1_H$ contains an unusual large insertion sequence (IS1) which is not found in the other ClpP proteins. The three smaller ClpP1 variants are generated by posttranslational multistep processing of $ClpP1_H$, from which the IS1 is cleaved, possibly during or immediately after the core assembly [44,57,58]. IS1 seems to protrude at the apical surface on the proteolytic core, possibly preventing the association with chaperones [44]. Consistent with this assumption, additional peripheral extrusions are observed on the typical barrel-shaped structure of the Clp core in [45]. The IS1-related sequence is found in the ClpP1 of Volvox as well, but not of other green algae such as *Nephroselmis olivacea* and *Ostreococcus tauri*, indicating its limited conservation [44].

5. The Clp chaperone expression, properties, oligomerization and interactions

Clp chaperones are members of the AAA^+ superfamily. The general function of this family is to trigger conformational changes in a broad range of protein substrates, thereby aiding in unfolding for degradation, refolding of aggregates, as well as disassembly of macromolecular protein-protein and protein-DNA complexes [59,60]. As outlined in Fig. 4, the typical domain architecture of proteins in this superfamily consists of an N-terminal domain (N-domain), which serves as a binding site for adaptor proteins and substrates, followed by one or two characteristic conserved modules, namely AAA domains or nucleotide binding domains (NBDs), each of which contains the well-known Walker A and B motifs required for ATP binding and hydrolysis, and the pore loop for substrate binding [60–64].

Plastid ClpC1/2 and ClpD belong to the class I Clp chaperones harboring two AAA modules. Arabidopsis ClpC1 and ClpC2 proteins are also known as Hsp93V (on chromosome V) and Hsp93III (chromosome III), respectively; we suggest to use the assignment ClpC1,C2 also to facilitate more easy species comparisons. Arabidopsis ClpC1 and ClpC2

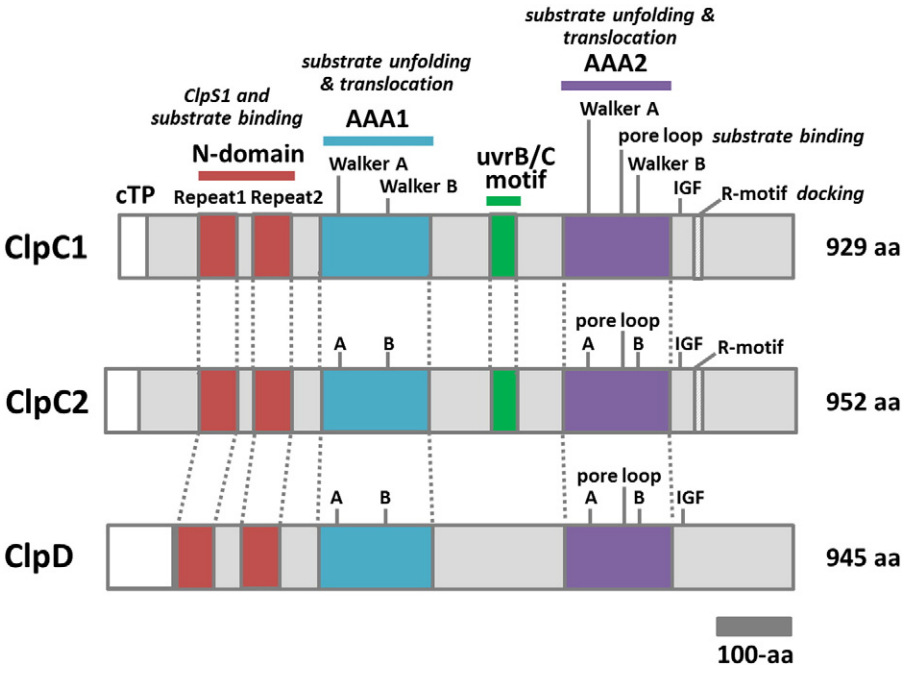

Fig. 4. Domain organization and functions of the ClpC1,2 and ClpD chaperones. Schematic shows ClpC/D primary structures. Clp chaperone proteins harbor of 1) chloroplast transit peptide (cTP, white), 2) N-domains (red) with two tandem repeat sequences for adaptor and substrate binding, 3) first ATPase domain (AAA1, aqua blue) containing Walker A and B motifs for substrate unfolding and translocation, 4) second ATPase domain (AAA2, purple) containing pore loop for substrate binding as well as two Walker motifs, and 5) IGF motif for ClpR-ring docking. ClpC1/2 but not ClpD contain an uvrB/C motif (green) with unknown function and an R-motif (shaded) for ClpR-ring association.

share around 90% sequence identity and more than 70% sequence identity with cyanobacterial ClpC, whereas ClpD shows only 45% identity to ClpC proteins in plastids and cyanobacteria. Some aspects of ClpC2 and ClpD have been biochemically characterized by using their recombinant proteins [65]. ClpC2 and ClpD proteins form homodimers in vitro (180 and 220 kDa, respectively) and also form higher-order assemblies, ranging from 500 to 700 kDa, in the presence of ATP, suggesting hexamerization. Both proteins have intrinsic ATPase activity. The kinetic parameters of ClpC2 are comparable to those of ClpA in *E. coli* or ClpC in cyanobacteria, and the affinity for ATP is consistent with its physiological concentration in leaf [65]. On the other hand, ClpD is kinetically slower compared to ClpC2, and the Km value for ATP is much higher than the [ATP] *in vivo*. ClpC2 and ClpD both show renaturation activity of a heat-aggregated model substrate, with ClpC2 having a higher efficiency. In addition, ClpC2 and ClpD are both capable of interacting with the N-terminal presequence (cTP) of the chloroplast protein ferredoxin [108]. Recombinant ClpC1 is unstable when expressed in *E. coli* [46,65]; consequently its *in vitro* properties have not been determined.

In vivo characteristics of Clp chaperones have been characterized in Arabidopsis. Clp chaperones are present in all plant organs, with highest abundances in photosynthetic tissues [46,66]. ClpC levels are higher in true leaves than in cotyledons and decline as the leaves become mature; this decline is more dramatic for ClpC2 than for ClpC1 [46,67,68]. On the other hand, ClpD levels increase in older leaves [67,68] and mRNA levels are strongly induced during leaf senescence [27,69]. Spectral counting analysis by mass spectrometry as well as immunoblot analysis showed that ClpC1 is several-fold more abundant than ClpC2 and ClpD [67,70]. ClpC proteins are present in the stroma mostly as dimers and in part as hexameric complexes, based on their native sizes from 200 to 600 kDa [2,46]. Given the relative amounts of two ClpC proteins, most if not all of the plastid chaperone complexes are assumed to be homo-oligomers. A small portion of ClpC1,2 are associated with inner envelope TIC components [71]. Cyanobacterial ClpC is observed as dimer, as well as in higher molecular weight complexes (669 to 1,500 kDa) [39].

Based on overwhelming evidence for bacterial ClpA-ClpP interactions, it is likely that binding of the Clp chaperone to the proteolytic core is also crucial for protein degradation in plastids. ClpA and ClpC (and ClpX) possess a conserved short hydrophobic motif in the C-terminus, the IGF/L motif or P-loop (Fig. 4), which binds to hydrophobic residues on the apical surface of the bacterial Clp protease core; this IGF domain is essential for the chaperone-core association in bacteria [28,72,73]. An additional short 8 amino acid sequence (rich in basic residues), called the R-motif, located just a few residues immediately downstream of the IGF loop, confers specific interaction between ClpC and ClpP3/R in cyanobacteria, and is also conserved in plastid ClpC1,2 [74], but not ClpD (Fig. 4). Several sequence motifs important for the chaperone-core complex formation in cyanobacteria are present in the N-termini of ClpP3 (MPIG motif) and ClpR (tyrosine and proline motifs) [74]. Two members of the chloroplast R-ring, namely ClpP1 and ClpR2, also contains the MPIG motif in the N-terminus and they are both orthologs of cyanobacterial ClpP3 (Fig. 2B). The other three members of the ClpR ring (ClpR1,3,4) do not have this MPIG motif but have the tyrosine and proline motifs, consistent with the orthologous relationship between ClpR1,3,4 and cyanobacterial ClpR (Fig. 2B; see also Section 3). Thus the R-ring containing ClpP1 and ClpR proteins but not the P-ring with the other ClpP subunits likely provides a docking platform for Clp chaperones in plastids, as proposed earlier ([27,43,53]. Nonetheless, the structural basis for plastid Clp chaperone-core interaction is not fully understood. There are 3 older studies that reported observation for (ATP-dependent) interaction between ClpP1 and ClpC based on co-immunoprecipitation and/or partially overlapping chromatography elution profiles; this was before the ClpPR core was identified [75–77]. More detailed evidence for interaction between the 325–350 kDa chloroplast ClpPR core complex and ClpC1,C2 hexamers has yet to be provided. It appears that the ClpPR-ClpC interaction is highly transient and most likely requires bound substrate.

Because chloroplast ClpC proteins are frequently co-purified with inner envelope protein complexes (including the initial discovery in 1993; [23]), they have been suggested to be involved in protein import, either as part of the translocation motor or as a quality control system – see [10,71]. The chloroplast inner envelope proteins Tic110 and Tic40 are implicated as a motor in protein translocation together with ClpC proteins, whose activity for ATP hydrolysis is stimulated by the

Tic40 C-terminal domain [78]. ClpC was suggested to assist in ATP-dependent Tic110 recruitment to the inner envelope as well [79]. Several subsequent elegant studies have shown that stromal Hsp70 is the major driving force in the energy-dependent protein translocation [80], inferring that ClpC is only a minor constituent in this process [81,82]. Notably neither Tic110 nor Tic40 are components in the recently-identified, general TIC complex of 1-MDa containing Tic20-I, Tic56, Tic100 and Tic214 [83,84] (see further Section 9).

6. Visible consequences of loss of Clp functions for development, growth and reproduction

Eubacterial Clp machineries are dispensable under normal growth conditions, while the cyanobacterial ClpP3/R complex, but not the ClpP1/P2 complex, is essential for cell viability [85,86]. Over the last decade, reverse genetic studies for most of the Arabidopsis nuclear-encoded ClpPR subunits, all three Clp chaperones and ClpS1 [42,43,46,87–93], and plastid-encoded ClpP1 in tobacco [26,94] and *C. reinhardtii* [95,96] have demonstrated the involvement of the Clp system in embryogenesis, plant or cell growth, plastid biogenesis and plastid proteostasis. Double and triple Arabidopsis Clp mutants clarified protein redundancies as well as bottle-necks in Clp function. The next Section (7) will summarize the molecular phenotypes most of which are based on proteome analyses, but here we first summarize conclusions for growth and developmental phenotypes.

In case of the ClpPR subunits, the strongest phenotypes are observed for those Clp proteins with 2 or 3 copies per complex (ClpP1, ClpP4 and ClpP5). Loss of ClpP4 or ClpP5 results in embryo developmental arrest at the globular stage; consequently seeds are very small and shriveled and cannot germinate, even with added sugars. In those species where targeted gene inactivation of plastid genes is possible (tobacco and *C. reinhardtii*), ClpP1 null mutants could not be recovered, indicating that also ClpP1 is essential for viability [26]. Down-regulation of ClpP1 at a later stage of development, after establishing a viable plant or viable cells, resulted in rapid loss of plastid function and loss of viability and cell death [94,96]. Null alleles for ClpR2, ClpR4 and ClpP3 (each 1 copy per core complex) each have delayed embryogenesis, white embryos and smaller seeds than wild-type plants [88,89,91]. Seeds of these null alleles can germinate under autotrophic conditions; however seedling development remains arrested in the cotyledon stage. Only when sugars are added to the medium do these mutant alleles develop true leaves. In case of ClpR2 and ClpR4 null mutants, such heterotrophically grown plants do not form any viable seeds; in contrast ClpP3 null mutants do slowly green, flower and eventually (after ~ 6 months) produce viable seed [91]. No null mutants for ClpP6 are available but we expect a similar phenotype as for ClpP3 [43]. ClpP6 antisense lines show a pale-green phenotype that is alleviated during leaf maturation [42]. Loss-function ClpR1 alleles are very different than the other ClpPR mutants and only have relative weak virescent phenotypes with normal fertility [89,93]. This moderate phenotype is due to partial functional substitution by ClpR3. No null mutants for ClpR3 are available and loss-of-function alleles could have similar phenotypes as either of the ClpR1,2,4 mutants. Finally, double mutants for ClpPR genes (*clpr1xclpr2-1*; *clpp3-1xclpr2-1*) show strong synergistic phenotypes, including those involving ClpR1, despite its relatively weak, but still easily visible, phenotype [89,91]. These observations show that most, if not all of the plastid Clp core subunits make distinct structural and/or functional contributions. Single mutants for each of the ClpT1,T2 are seemingly normal in appearance [53], but the double mutant shows a visible phenotype (Kim et al., in preparation). The double mutant is not embryo or seedling lethal as previously stated [53].

The ClpC1 null allele shows a pale green phenotype throughout all developmental stages [97,98], while ClpC2 and ClpD null alleles has no visible phenotype [46,99]. This continued pale green phenotype even in older leaves clearly contrasts the developmentally controlled leaf phenotypes in the viable ClpPR mutants; the reasons for this opposing developmental impact are not clear. Complete loss of both ClpC proteins results in embryo lethality, but overexpression of ClpC2 complements the ClpC1 deletion phenotype [100]. Also, given their high sequence identity, these observations indicate that ClpC proteins share essential functions. The distinct visible phenotypes between the two ClpC1,2 loss-of function mutants can be largely attributed to their different accumulation levels. Consistently, ClpC2 levels are increased in ClpC1 loss-of-function alleles [46,67]. ClpD gene expression is under the control of a zinc finger homeodomain transcriptional activator that is induced by drought, high salinity and abscisic acid and specifically binds to the *CLPD* promoter region containing a dehydration-inducible cis-element [101]. ClpD protein accumulates not only in senescing leaves but also in younger leaves and ClpD involvement and function in plastid proteome homeostasis awaits further investigation. The ClpS1 null mutant (*clps1*) has no obvious visible growth phenotype, did not show genetic interaction phenotypes with ClpR2, ClpC1,2 nor ClpD, but its chlorophyll content is slightly decreased [46]. However, *clps1* is more susceptible than wild-type to the chloroplast translation elongation inhibitor chloramphenicol, but not to the cytosolic translational inhibitor cycloheximide, suggesting a possible functional link between plastid translation (elongation) and ClpS1-directed proteolysis.

7. Clp loss-of function proteome phenotypes

The function of the Clp protease system lies in the selected degradation of plastid proteins and perhaps also degradation of protein fragments generated by other plastid proteases. Whereas proteases (also) can play a role in maturation and activation of proteins [14], there is no evidence for such a role for the Clp system in organelles or prokaryotes. In fact, the molecular cleavage mechanism and the trapping of unfolded substrates in the chamber of the Clp protease core makes a role in maturation/activation unlikely.

To better understand the physiological significance of the Clp system in plants, large scale quantitative proteomics has been applied to analyze steady state proteomes of loss-of-function mutants in different Clp components, namely ClpR2 [90], ClpR4 [89], ClpP3 [91], ClpS1, ClpC1, the ClpS1x ClpC1 double mutant [46], as well as ClpT1 x ClpT1 double mutants (Kim et al., in preparation). These studies used 1D-gel separation followed by high-resolution tandem mass spectrometry and quantification by spectral counting, and in case of ClpR2 also quantification using the stable isotope labeling technique cICAT [90].

Following statistical analyses, these studies found a very consistent pattern of up- and down-regulated proteins for mutants of ClpPR core subunits. Collectively, a clear chloroplast phenotype emerges from these Clp core mutants with nine key characteristics summarized in Figure 5: i) a strong loss of photosynthetic capacity through systematic loss of the thylakoid-bound photosynthetic machinery and the Rubisco holocomplex; this is consistent with its pale-green phenotype and delayed growth, ii) strong differential up-regulation of plastoglubular proteins, in particular of module 2 of the plastoglobular co-expression network [102], indicative of a thylakoid membrane homeostasis problem, iii) increased accumulation of a subset of DNA/nucleoid interacting proteins most likely involved in DNA/genome quality control, iv) differential effects on proteins involved in RNA processing, v) strong upregulation of protein translation factors and tRNA synthases, but not plastid ribosomes, vi) systematic up-regulation of all stromal chaperone systems and the ClpB3 unfoldase, vii) upregulation of the chloroplast Sec machinery suggesting a bottleneck in thylakoid protein insertion, viii) up-regulation of a narrow set of chloroplast proteases (EGY2, SPPA, PREP1, LAP2) as well as the stromal processing peptidase SPP, ix) increased levels of enzymes involved in primary and secondary metabolism, most of which can be explained by loss of ATP/NADPH production and delayed chloroplast development and x) a changes in

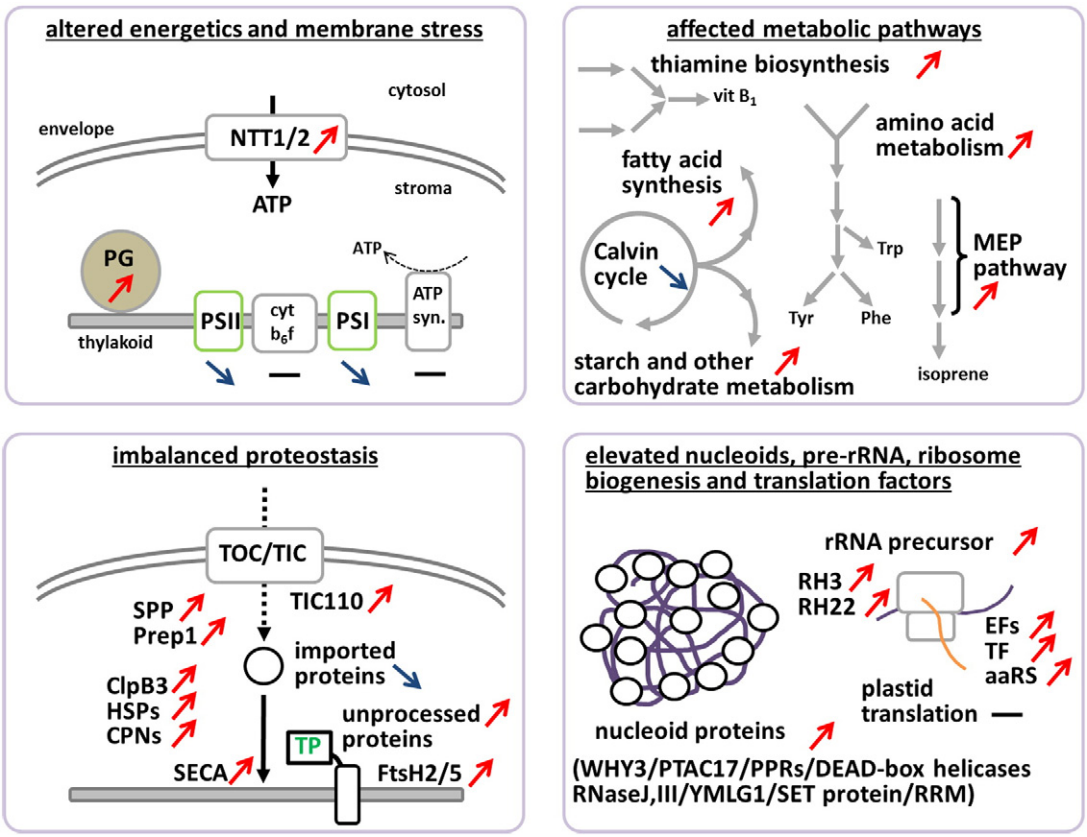

Fig. 5. Summary for proteome changes due to the loss of plastid Clp proteolytic capacity. Reduced Clp proteolytic activity causes 1) a significant decrease in photosystem (PSI and PSII) complexes resulting in loss of energetics, a possible compensatory increase in levels of nucleoside triphosphate transporters (NTT1/2) involved in ATP import, and elevated plastoglobule (PG) proteins indicative of membrane stresses, 2) decreased levels of Calvin cycle enzymes and increased levels of enzymes in shikimate pathway for amino acids, MEP pathway for isoprenoid, thiamine biosynthesis for vitamin B1, fatty acid synthesis, starch and other carbohydrate metabolism, 3) imbalanced protein homeostasis including inefficient protein import, unprocessed protein accumulation (*e.g.* LHCIIs and PsaF), upregulation of import machinery (*e.g.* TIC110), sorting machinery (*e.g.* SEC), processing peptidases (*e.g.* stromal processing peptidase [SPP] and presequence proteases1 [Prep1]), chaperones (*e.g.* HSP70s, HSP90, CPN60s, CPN21 and CPN10) and proteases (*e.g.* FtsH2/5), 4) overaccumulation of nucleoid proteins, ribosomal RNA (rRNA) precursors, ribosome biogenesis regulators (*e.g.* RH3/22) and translation factors (elongation factors [EFs], trigger factor [TF] and aminoacyl-tRNA synthetases [aaRS]) without a significant loss of translation. Increased and decreased protein levels are indicated in red and blue arrows, respectively.

envelope transporters, such as the up-regulation of the inner envelope ATP/ADP translocators (NTTs) which import cytosolic ATP into the chloroplast, confirming the reduced ATP-generating capacity in the chloroplast [103].

It is important to point out that the core mutants did not show a systematic defect in accumulation levels of chloroplast-encoded proteins in the core mutants, even if the mutants show various inefficiencies in RNA processing and upregulation of RNA maturation factors and translation factors. Only chloroplast-encoded proteins that are part of the photosynthetic apparatus were down-regulated, whereas chloroplast-encoded ribosomal proteins were not affected. This likely reflects a systematic down-regulation of the photosynthetic apparatus, including nuclear-encoded proteins. The mechanism for this selective loss of accumulation of chloroplast-encoded protein is unclear but could be i) accelerated turnover of thylakoid proteins, ii) retrograde signaling specifically resulting in down-regulating expression of nuclear-encoded thylakoid proteins (see Section 14) or iii) reduced efficiency of protein import of photosynthetic proteins (see Section 9). A number of up-regulated proteins likely represent direct targets of the Clp system, but these steady state proteome data do not easily allow us to recognize such candidate substrates. In fact, we believe that most of these up-and down regulated proteins represent secondary effects of the loss of Clp function or Clp capacity. Nevertheless, this proteome phenotype suggests that the Clp protease system likely has broad substrate specificity and that its function is essential for chloroplast biogenesis and cannot be replaced by other proteases. Importantly these studies show that the consequences of the loss of Clp core capacity are very similar in the different ClpPR core mutants, irrespective of which ClpPR subunit was underexpressed. This demonstrated that whereas the individual ClpPR proteins make important or essential contributions to the Clp complex, each of them unlikely have a specific role in substrate selection.

Extensive quantitative proteome analysis of the pale-green ClpC1 null mutant, showed down-regulation of enzymes for photosynthetic electron transport, primary carbon metabolism including Calvin cycle, glycolysis and photorespiration and up-regulation of proteins involved in protein biogenesis and plastid gene expression, consistent with the yellow leaf phenotype, reduced growth and the role of ClpC1 in import [46]. The more modest *clps1* proteome phenotype is in line with the proposed function of ClpS1 as a nonessential adapter for the Clp system and pointed to destabilization of the tetrapyrole pathway in addition to a few 'scattered' effects. Interestingly, the proteome of the *clpc1xclps1* phenotype suggested a ClpS1 and ClpC1 interaction effect on plastid gene expression components and nucleoid interactors, including RNA processing and editing, as well as 70S ribosome biogenesis [46].

8. Accumulation and assembly of ClpPR proteins in Clp mutants

The reverse genetics studies in Arabidopsis have shown that each ClpPR core subunit (except for ClpR4 for which there is no mutant analysis) makes a specific contribution to ClpPR core function and demonstrated that those present in 2 or 3 copies per complex (ClpP4, ClpP5 and ClpP1) are essential for embryogenesis. However, the phenotypes for those present in one copy shows variation, which in case of ClpR1 can be explained by partial redundancy with ClpR3 [89,93].

Careful analysis of mRNA levels for the complete Clp family in the *clpr4-1* null mutant (no detectable *CLPR4* mRNA) showed that mRNA levels for all ClpP/R/S/T/C/D genes decreased by 20-30%, except ClpC2 (up by ~8%) [89]. It thus appears that there is only modest transcriptional regulation when there is an unbalance between accumulated ClpPR subunits. There is no straightforward explanation for the subtle phenotypic differences between the null mutants for ClpP3 and ClpR2/R4 (ClpP3 but not ClpR2/R4 null mutants could slowly grow on soil after an initial phase on sucrose [89]). However, immunoblotting and/or MS-based quantification in older ClpP3 rosettes showed increased levels of ClpP4, ClpP6, ClpP1, ClpP5, suggested alternative ClpPR cores did provide some functionality but likely with limited protease capacity. Such compensatory effects did not take place in the ClpR2, ClpR 4, nor ClpP4 and ClpP5 null alleles. Finally, in the viable ClpPR lines, the leaf phenotypes gradually improved likely due to reduced necessity for Clp protease capacity once chloroplast biogenesis was complete.

The assembly state of the ClpPR core in ClpPR null mutants (in ClpR2, ClpR4, ClpP3) or underexpressors (antisense ClpP6 and the leaky *clpr2-1* mutant) has been assessed using native gels followed by immunoblotting [42,91], MS-based quantification using the iTRAQ reagents [88], or using absolute quantification using spike-in peptides generated from QConCat constructs [91]. The collective outcome of these assembly studies is not completely straightforward but does generally suggest that the full (325–350 kDa) core assembly is strongly reduced and that instead subunits mostly accumulate in 180–200 kDa assemblies. Furthermore, as mentioned above, 325–350 kDa cores with modified composition appear to accumulate in some of the lines, most likely with reduced catalytic efficiency.

9. The role of the Clp system in proteostasis

Whereas the Clp chaperones and ClpPR cores are soluble protein complexes located in the stroma, a minor portion of ClpC, but not ClpD, is localized at the inner envelope membranes interacting with the protein translocation machinery [104–107]. Also a small percentage of ClpPR cores are attached to the envelope membranes [67]. ClpC chaperones have intrinsic abilities to bind the N-terminal cTP as well as to refold proteins *in vitro* [65,108] and *in vitro* chloroplast protein import efficiency for a number of precursors was reduced in the ClpC1 and ClpR1 mutants [67,109]. This suggests that the Clp protease participates in protein quality control of incoming nuclear-encoded proteins, such as the degradation of trapped, damaged or aggregated proteins at the inner envelope import machinery– see for discussion [71].

Reduced Clp protease core capacity, and to a lesser extend loss of ClpC1, results in strong up-regulation of the stromal protein folding machineries, including Cpn60/21/10, Hsp70 and Hsp90, as well as the unfoldase ClpB3 [89–91]. This suggests that loss of Clp capacity results in protein aggregation and upregulation of unfolding capacity. In particular ClpB3 is highly (5-10 fold) upregulated in the ClpPR core and the ClpC1 null mutants. ClpB3 lacks the essential IGF domain for interaction with the Clp core and is not part of the Clp protease system [29]. ClpB3 homologs in bacteria have shown to participate in the unfolding and subsequent reactivation of aggregated proteins aided by the DnaK (HSP70) chaperone system [110,111] and plastid ClpB3 is important for plastid development and heat stress responses [112]. The aberrant thylakoid structure in the ClpR4 null mutant can be partially restored under very low light in the cold (4 °C) where protein expression and folding slow down, suggesting that the Clp protease is important in removal of toxic protein aggregates [89]. The observed genetic interaction of ClpR2 with ClpB3 further supports this function of the Clp system [90].

ClpC1 genetically interacts with cpSRP54 (for Chloroplast Signal Recognition Particle 54) that is involved in co- and post-translational sorting of thylakoid proteins, and ClpC1 up-regulation is observed in developing leaves of the SRP54 single null mutant [113]. Defects in sorting can result in protein mislocalization, which then requires degradation of the mislocalized protein providing an explanation for the genetic interaction effect between SRP54 and ClpC1. Furthermore unprocessed PsaF and LHCII proteins are incorporated and accumulated in thylakoids of the *clpr2-1* core mutant [88]. Processing of the preprotein is not necessarily required for intraplastid protein sorting [114,115], but accumulation of unprocessed proteins could disrupt thylakoid integrity [116,117]. A fraction of the ClpPR core and ClpC1,2 population have been shown to be recruited to thylakoids in case of the Clp core mutants as well as the thylakoid FtsH2 (VAR2) mutant [88,118]. Indeed the ClpPR protease core complex in Arabidopsis chloroplasts was initially discovered during analysis of the peripherally-associated thylakoid proteome [25]. Recently, it was shown that the Arabidopsis ClpC chaperones and the ClpPR core is required for degradation of the thylakoid copper transporters PAA2, whereas the thylakoid FtsH protease and lumenal and stromal DEG proteases were not required [119] Furthermore, ClpP1 in Chlamydomonas has been shown to contribute to removal of unassembled/misassembled thylakoid cyt*b6f* subunits [95]. Together this demonstrates that the Clp protease system also helps to maintain proteostasis in the thylakoid, thus complementing the activity of thylakoid proteases.

10. Clp substrates and molecular functions

The most direct method to understanding the function(s) and significance of the Clp system in plastid is to identify its substrates (Fig. 6). So far two different approaches identified substrates for the Clp system. The first study identified stromal proteins that specifically interact to ClpS1 [46]. The second study systematically screened the conditional stability of a specific chloroplast protein in various plastid protease mutants [119].

ClpS1 interactors are involved in nucleoid maintenance/organization and metabolic pathways Affinity purification using GST-ClpS1 affinity columns incubated with stromal proteomes from wt and null mutants in ClpS1, ClpC1 and the ClpS1 x ClpC1 double mutant followed by extensive mass spectrometry and immunoblotting analyses identified multiple ClpS1 interactors [46] (Fig. 6). Furthermore, the use of GST-ClpS1 proteins in which the conserved ClpS substrate binding site was mutated confirmed the highly selective interaction for several proteins. A similar strategy was successfully used for *E. coli* [120]. The specific plastid ClpS1 interactors proteins include enzymes associated with the nucleoid, secondary metabolism for aromatic amino acids and a central enzyme in tetrapyrrole biosynthesis, as well as other proteins with unknown function.

A subset of ClpS1 targets is associated with plastid nucleoid maintenance and organization. RADiation sensitive52-2 (RAD52-2) is involved in plastid genome maintenance [121] and is localized in the nucleoid [122]. PTAC17 was originally discovered as a component of the plastid transcriptionally active chromosome within nucleoids [123,124]. In particular PTAC17 is found to be overaccumulated in ClpC1 and Clp core mutants but not in the ClpS1 knockout [46,91], suggesting that Clp system is required for PTAC17 degradation but that ClpS1 is not essential for PTAC17 recognition. PTAC17 expression is higher in proplastids than in fully-developed chloroplasts, and furthermore nucleoid protein abundance is generally decreased as the chloroplast develops [9]. The nucleoid protein accumulation is thus negatively correlated with Clp expression, raising the possibility of Clp participation in nucleoid degradation during plastid development. In fact multiple other nucleoid proteins also overaccumulated in the Clp core mutant [91]. Now that RAD52-2 and PTAC17 have been identified as ClpS1 targets, other nucleoid proteins could be considered as promising targets for the Clp system.

A subset of the likely substrates is captured by ClpS1 with strict dependency on its two amino acids that correspond to N-degron binding residues in bacterial ClpS [46]. One such protein is glutamyl-tRNA reductase (GluTR). GluTR is the initial enzyme in tetrapyrrole biosynthesis where it catalyzes the rate-limiting step converting glutamyl-tRNA (Glu-tRNA) to glutamate-1-semialdehyde and also serves as the hub of multitude posttranslational regulations [125]. Glu-tRNA is a substrate not only for GluTR in the tetrapyrrole pathway but also required for plastid translation, setting up a competition for Glu-tRNA between these

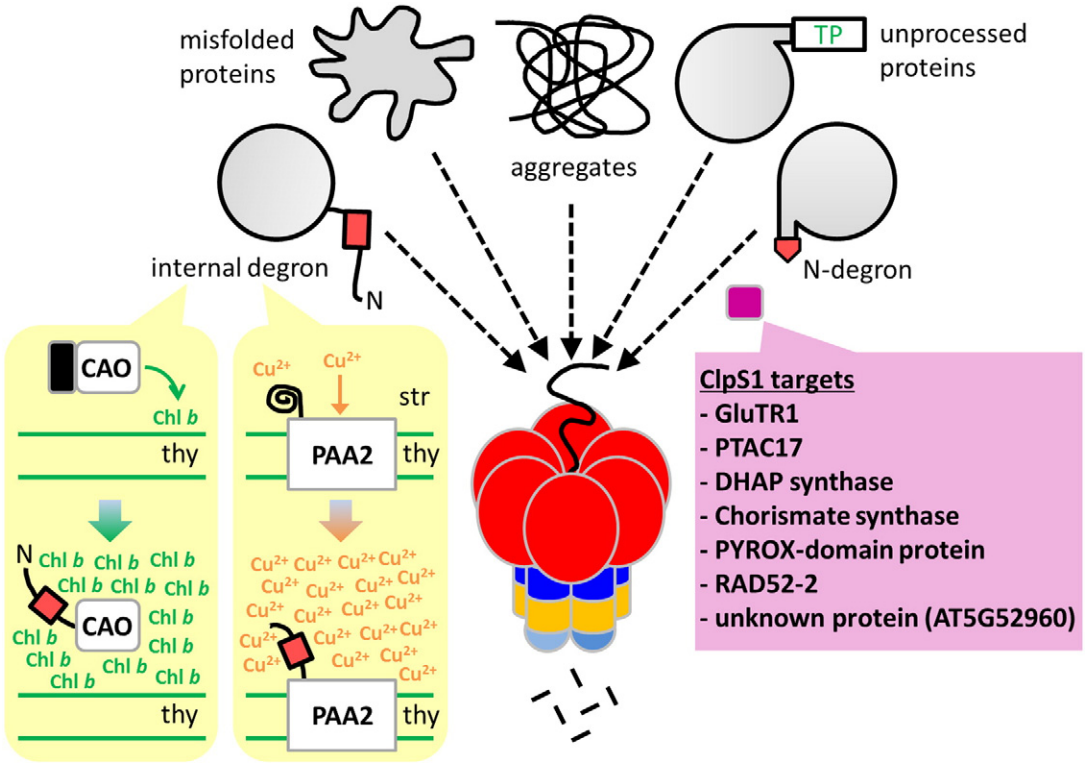

Fig. 6. Suggested degradation pathways for different types of substrates. Plastid Clp system is suggested to degrade five types of substrates: 1) proteins with internal degrons that are intrinsically buried within the interior but exposed upon conformational changes induced under certain cellular stimuli (*e.g.* high concentrations of chlorophyll b [chl b] for chlorophyllide a oxygenase [CAO] or copper [Cu^{2+}] for Plant ATPase of Arabidopsis2 [PAA2]), 2) misfolded proteins, 3) aggregates, both of which could be generated through protein translocation across the envelope membrane, 4) unprocessed proteins accumulating in thylakoids, 5) proteins having N-terminal destabilizing residues (N-degrons) that are potentially recognized by ClpS1 whose targets identified so far are indicated. Degrons are shown in red. TP, transit peptide.

two major pathways. Given the chloramphenical sensitivity in the ClpS1 mutant, we proposed that ClpS1 helps regulate the balance between these pathways by shifting the Glu-tRNA flux towards translation through accelerated GluTR degradation. Furthermore, given the ClpC1-dependent degradation of chlorophyll a oxygenase (CAO) and its specific internal degron [126,127] (see Section 11) (Fig. 6), a multilayered tetrapyrrole pathway regulation involving the Clp system is plausible. ClpS1 targets also include 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase and chorismate synthase (CS). DAHP synthase and CS catalyze, respectively, the first and the final reactions in the shikimate pathway providing precursor for aromatic amino acids [128]. In particular CS has been observed to be down-regulated during etioplast-to-chloroplast transition, when the Clp machinery is rather up-regulated [7]. Given that the CS level is increased in null mutants for ClpS1 and ClpC1 [46], plastid Clp protease is likely responsible for CS degradation during etioplast dedifferentiation.

ClpC1 and the Clp protease core degrade a transporter in copper homeostasis The thylakoid-located copper transporter, PAA2/HMA8 (P-type ATPase of Arabidopsis2/Heavy-metal-associated8), mediates copper delivery to the lumenal cuproprotein plastocyanin for photosynthetic electron transport [129] and is down-regulated through protein degradation under high copper conditions [130]. Systematic screening of protein stability of PAA2 in various plastid protease mutants identified the ClpPR core and ClpC1 as essential components to degrade PAA2 (Fig. 6). In contrast, neither ClpS1, nor several thylakoid or stromal proteases (FtsH, SPPA, DEG) influenced stability of PAA2 [119]. Degradation of PAA2 was induced by providing the plants with sufficient copper in the growth medium. The copper-dependent PAA2 degradation was abolished in the *clpr2-1* mutant but unaffected in the mutants for the other chloroplast proteases such as FtsH, Deg, SPP and PREPs [119]. PAA2 deregulation was also found in the ClpC1 knockout but not in ClpC2 or ClpD mutants, consistent with ClpC1 being the major Clp chaperone in plastids. In addition, the copper-induced PAA2 response is normal in the ClpS1 mutant, indicating that ClpS1 is dispensable for this PAA2 lifetime control. A copper-driven conformational change in PAA2 is proposed to be the trigger for substrate recognition by the Clp system. This finding thus identifies the first cofactor-induced protein degradation by the plastid Clp protease and establishes Clp as a protease (also) required for degradation of a thylakoid protein. Such systematic screening of protease mutant for stability (or lack of degradation) of proteins for which the half-life is known to be (easily) influenced by external signals is an attractive tool for protease substrate discovery.

11. Clp substrate selection and delivery mechanisms; ClpS, the N-end rule and more

Selective protease substrate recognition and delivery are crucial for ensuring optimal levels of functional proteins, eliminating dysfunctional proteins but avoiding unwanted proteolysis. A protein that is marked for degradation must harbor a degradation signal, assigned a degron. Degrons can be generated through post-translational modifications of the substrate protein, including i) enzymatic addition of an amino acid by an amino-acid transferase, ii) proteolytic removal of one or more amino acids from the N- or C-terminus thus exposing a degron, iii) specific chemical modification(s) of specific amino acid residues (*e.g.* acetylation, phosphorylation or oxidation). Alternatively, conformational changes in the protein can result in exposure of a degron that is otherwise intrinsically buried within the structure; such

conformational changes can include change association/dissociation of interacting proteins, peptide, metabolite or cofactors [131]. Each of these mechanisms are relevant for Clp substrate recognition and delivery in non-photosynthetic bacteria and perhaps also for photosynthetic eukaryotes.

Clp degrons are best studied in *E. coli,* in particular substrates containing the N-terminal degradation signal, called the N-degron. Degradation of N-degron substrates follows the N-end rule that relates the regulation of the *in vivo* half-life of a protein to the identity of its N-terminal residue (reviewed in [132]). This pathway was originally discovered in eukaryotic ubiquitin-mediated proteasome system and is highly conserved in many organisms [133,134]. Due to the lack of the *bona fide* ubiquitin-proteasome system, bacteria utilize an N-end rule pathway that instead requires the Clp machinery for degradation. Bacterial N-terminal destabilizing residues are classified into two groups, namely primary and secondary destabilizing residues (reviewed in [131]). Secondary destabilizing residues are basic amino acids (Arg and Lys), Met and acidic amino acids (Asp and Glu), each of which serves as a signal for the posttranslational modification of a primary destabilizing residue (Leu or Phe) onto the N-terminus of the target protein by leucyl/phenylalanyl-tRNA-protein transferase (LFTR) for basic amino acids and methionine or by leucyl-tRNA-protein transferase (BPT) for acidic amino residues. Alternatively, primary destabilizing residues can be generated through internal cleavage by proteases, as was suggested by MS-based analysis of trapped ClpS substrates [135]. Primary destabilizing residues (Leu, Phe, Tyr and Trp) are directly recognized by the bacterial adaptor protein, ClpS, which then delivers N-degron substrates to the ClpA chaperone for proteolysis. Notably, ClpS is suggested to mediate a directional translocation of N-degron substrates from N- to C-terminus for facilitating substrate access to the chaperone pore, where a hydrophobic motif within an unstructured N-terminal region rather than the very N-terminus on the substrate serves as a chaperone-recognizing site [136]. A staged delivery model of N-end rule substrates was proposed [137]. A recent biochemical study suggests that ClpA pulls on the unstructured N-terminal extension of ClpS to trigger substrate delivery [138].

Plastid ClpS1 is present both as a monomer and as part of higher molecular weight complexes of ~600 kDa, similar as hexameric ClpC chaperones. Consistently, physical interactions between ClpS1 and ClpC1/2 were observed, suggesting that the bacterial ClpS-ClpA substrate delivery system is conserved in chloroplasts [46]. ClpS1 accumulation in leaves is controlled in a spatiotemporal fashion, with the highest levels during leaf expansion followed by rapid decline upon senescence. This suggests that ClpS1-directed proteolysis functions in particular during early chloroplast development. Based on primary sequence comparisons, the residues for N-degron binding and substrate specificity in bacterial ClpS are generally retained but not identical in ClpS1, and thus the canonical substrate recognition mechanism for the N-degron seems not to be perfectly conserved in chloroplasts. Furthermore plant homologs for the E. coli aminotransferases LFTR and BPT have (so far) not been found in chloroplasts [139].

Through systematic tobacco chloroplast transformation the (de)stabilizing effect of each 20 amino acids in the penultimate position (immediately downstream of the formyl-methionine) was analyzed [139]. Based on these experiments, it was proposed that the major determinant for protein stability of chloroplast-encoded proteins resides in the N-terminal region [139]. It was suggested that Cys and His are N-terminal destabilizing signals in chloroplasts [139]. However, a role for the Clp system in the context has not been tested and it is not clear if the same stability rule apply to nuclear-encoded and plastid-encoded proteins. Preliminary results using so-called degradomics techniques suggest that indeed different rules apply (Rowland and van Wijk, in preparation)

The first described substrate for the bacterial Clp system is its own chaperone subunit, ClpA. ClpA is degraded by ClpAP in the absence of substrates or in the presence of excess ClpA compared to ClpP [140]. Such autodegradation has been proposed to be a mechanism regulating the *in vivo* level of ClpA and is negatively regulated by ClpS [141]. Although how ClpS inhibits ClpA autodegradation remains elusive, the last 9-residues located in the flexible C-terminus of the second AAA domain of ClpA is shown to serve as the signal [142]. Cyanobacterial ClpC is unlikely regulated by autodegradation, since an excess amount of the chaperone is not degraded by the proteolytic core in experiments of purified ClpC and ClpP3R [56], while the existence of ClpC autodegradation in chloroplasts is not known. However, given that ClpC proteins are stable in the mutant lacking ClpS1 [46], ClpC autodegradation is unlikely to be under negative regulation by ClpS1. ClpC proteins are down-regulated in Clp mutants lacking ClpP3 or ClpR4 [89,91] but are up-regulated in core mutants with much weaker phenotype (ClpR1 or the leaky ClpR2 mutant) [67,90]. Thus there appears to be a mechanism regulating ClpC levels in response to the amount of functional ClpPR core complex.

When ribosomes fail to complete or terminate protein synthesis properly, they stall on the mRNA with incomplete nascent polypeptide chains that might be toxic if released (reviewed in [143]). In bacteria such stalled nascent chains are tagged at their C-termini by an 11-residue peptide named SsrA or tmRNA (for small stable RNA or transfer-messenger RNA). These SsrA-tagged polypeptides are generated through *trans*-translation [144]. ClpA recognizes SsrA-tagged proteins for degradation, which is competitively inhibited through ClpS binding to the ClpA N-domain [141]. ClpXP is the primary acceptor for SsrA-tagged substrates in bacteria, and homologs are present in plant mitochondria but not in chloroplasts. SsrA sequences are present in plastid genomes of some red and green algae [145] but are not found either in plastid or mitochondrial genomes of higher plants. Thus some algal species but not land plants might retain an SsrA-tagging system in plastids. It is not known how plastids clear stalled nascent chains and if Clp is involved.

Cyanobacterial ClpC can hold unfolded proteins in a non-aggregated state to prevent aggregation and otherwise bind to aggregates for refolding, all of which are ATP-dependent reactions [146]. This intrinsic disaggregating ability of ClpC contrasts with ClpA requiring ClpS for aggregate recognition and disaggregation [147]. Cyanobacterial ClpS1 can interact with ClpC but has no reported effects on the chaperone activities [146]. Plastid Clp chaperones, as described above, also show the ability for disaggregating/folding activity; the effect of ClpS1 on this activity has not been tested [65]. In addition the Clp chaperone binding ability to a cTP-containing protein suggests that the Clp system could recognize unprocessed proteins as well.

Plastid Clp protease is proposed to regulate the accumulation level of CAO, since the loss of ClpC1 causes CAO overaccumulation [127]. CAO influences the antenna size of photosystems through chlorophyll *b* synthesis [148]. CAO stability is under negative feedback regulation involving chlorophyll *b*, and the N-terminal domain (referred to as the A domain) is necessary for this regulation [149,150]. Serial deletion analysis of the A domain has identified a 10-residue sequence (97-QDLLTIMILH-106) as an in vivo determinant for CAO stability [126]. Surprisingly, in silico prediction suggests that there seem to be 1,343 sequences similar to this degron. Of these potential degradation signals, a sequence (9-GRLLAVHIMH-18) in the N-terminal region of CP47, a plastid-encoded chlorophyll-binding subunit of PSII, was also experimentally shown to serve as a degron. Whether and how those two degrons are recognized by Clp chaperones or other protease systems deserves further study.

12. Clp proteolysis in crop species

The biochemical function and physiological importance of the plastid Clp protease have mainly been studied in the dicotyledon Arabidopsis but is poorly understood in monocots, including the crop plants rice and maize. Research on this proteolytic machinery in crop species deserves a high priority for improving future agricultural productivity and sustainability. Two recent studies describe mutant phenotypes for

plastid-localized ClpP homologs in rice and maize; these closely resemble the phenotype of their Arabidopsis homologs. A ClpP6 rice loss-of function mutant showed a virescent yellow leaf phenotype, where the chlorotic leaves gradually turn green in later developmental stages [151]. Consistently, rice CLPP6 transcript levels are most abundant at the early chloroplast and leaf developmental stage. While the composition of rice Clp protease cores have not been determined, yeast-two-hybrid testing the binary interactions between plastid ClpP proteins suggested interactions between ClpP3 and ClpP6, ClpP4 and ClpP5, ClpP6 with itself [151]. An independent forward genetics study identified another virescent rice mutant in ClpP6 – however, no gene identifier was provided [152]. Maize mutants for two ClpP5 paralogs were isolated through a gene-background interaction study with two different inbred lines B73 and PH09B [153]. A B73 mutant with an insertion in one CLPP5 (Chr.9_ClpP5) shows a virescent yellow-like phenotype with altered size and structure of the chloroplast that recovers and becomes indistinguishable from the wild-type as the plant grows. A Mu insertion line in PH09B for another CLPP5 gene (Chr.1_ClpP5) did not show a visible phenotype. However, loss of both copies of the CLPP5 genes causes lethality, reminiscent of ClpP5 knockout alleles in Arabidopsis [89]. These observations support the importance of Clp dependent proteolysis in the monocot crops maize and rice.

13. Clp as part of the proteolysis network

In addition to the Clp protease system, non-green plastids and chloroplasts contain additional proteases; together these proteases must control plastid proteostasis. A number of these proteases have been characterized at various levels of detail, whereas other proteases have not been studied at all - reviewed in [13,22]. Whereas each of these proteases may have specific targets (substrates) and recognition mechanisms, it is very likely that subsets of proteases have overlapping substrates and/or act sequentially on the same substrate(s). Relatively little is known about such functional interactions and overlap in plastids, except for a few cases. The first case is the step-wise degradation of the D1 reaction center protein of Photosystem II located in the thylakoid membrane. The D1 protein has five trans-membrane domain and it is cleaved in the soluble domains by the activity of soluble DEG proteases in the thylakoid lumen and stromal side of the thylakoid, followed by processive degradation by the thylakoid- bound heteromeric ATP-dependent FtsH protease, consisting of FtsH1, FtsH2 (VAR2), FstH5 and FtsH8. Clp chaperone and proteolytic core both are up-regulated and recruited to the thylakoid membrane in the mutant lacking FtsH2, suggesting that they perhaps they can share some of the thylakoid FtsH substrates [118]. Furthermore, molecular genetics-based experiments in *C. reinhardtii* suggested that thylakoid FtsH is involved in degradation of cytochrome b_6f complex together with Clp proteins (Wei et al., 2014; Malnoë et al., 2014); however molecular details of substrate selection and degradation are entirely lacking. SPP is responsible for pre-protein processing through cTP cleavage [154]. Degradation of these cleaved cTP likely involves two metallo-endopeptidases (M16 family), PREP1 and PREP2 [155,156] as well as OOP, a metallo-oligopeptidase in the M3 family [15]. It has been postulated that also the Clp protease can contribute to cTP recycling [27]. A Lon protease family member, namely Lon4, is also an ATP-driven serine protease and functions in leaf mitochondria and chloroplasts [157]. Bacterial Clp and Lon proteases degrade common substrates such as ssrA-tagged proteins and TrfA replication initiation factor in a complementary fashion [158,159]. Although ssrA tagging and TrfA homologs appear absent in higher plants, Lon4 and Clp protease could perhaps functionally complement each other also in plant mitochondria and plastids. Suppression analysis of the variegated *var2* phenotype uncovered that loss-of-function mutants in both ClpPR core (ClpR4) and Clp chaperone (ClpC2) proteins suppress the *var2* phenotype [99,160–162]. However, it is important to realize that these suppression mutants retain their reduced growth phenotype but lack the variegated pattern, indicating that these suppressors do not substitute for the FtsH2 function.

14. Integration of plastid Clp function with the cell; feedback regulation and retrograde signaling

Retrograde signaling from plastids/chloroplasts to the nucleus is important to instruct the nuclear genome about the physiological state of the chloroplasts and adjust the chloroplast proteome composition as needed. Multiple retrograde pathways have been proposed. It appears that plastid gene expression, thylakoid redox state, reactive oxygen species, as well as metabolic pathways all contribute to retrograde signals. It is also clear that some of these intra-plastid signals can be integrated and drive the same signaling pathways, but also that separate signaling pathways exist [163–165]. As was discussed in Section 7, insufficient Clp capacity results in a number of physiological responses within the chloroplasts; these likely result in retrograde signaling events. Clear examples are the very strong overaccumulation of the chloroplast chaperone systems and the ClpB3 unfoldase in the various Clp loss-of-function mutants. It is not yet known how the increased levels of chaperones are accomplished;' this could be through increased life-time or increase in transcripts and/or translation outside the chloroplasts. Inducible down-regulation of ClpP1 in Chlamydomonas cells and concomitant analysis of the proteome and transcriptome showed that gradual depletion of ClpP1 resulted in multiple cellular defects and ultimately autophagy [96]. Based on this system analysis it was suggested that retrograde signaling is involved in plastid quality control, but no specifics were identified. Cell in *C. elegans* monitor mitochondria protein import efficiency of the transcription factor TFS-1, which has both a mTP and a nuclear localization signal. Normally ATFS-1 is rapidly imported into mitochondria and degraded by LON. However, mitochondrial stress or dysfunction results in reduced import efficiency, allowing some of the ATFS-1 to accumulate in the cytosol and subsequently traffic to the nucleus, where it adapts transcription to efficiently recover from mitochondrial dysfunction [166]. It is conceivable that a similar system operates in plastids, e.g. when the plastid Clp system has insufficient capacity (i.e. in mutants or under particular conditions). A DNA binding protein WHIRLY1 [167] and transcription factor HEMERA/PTAC12 [168] show dual location in chloroplast nucleoids and the nucleus. The regulatory mechanism for distribution of such proteins between these two locations could perhaps involve intra-plastid proteostasis events, including Clp driven proteolysis.

15. Conclusions, key questions and challenges

Research over the last decade has shown that the plastid Clp system greatly diversified and increased in its complexity as compared the Clp system in non-photosynthetic prokaryotes and its bacterial progenitor. Many aspects of the Clp functional organization, including the subunit stoichiometry of the ClpPR core have been determined. Multiple lines of evidence demonstrate that the Clp system is essential for chloroplast biogenesis and proteostasis, including for plastids during embryogenesis. Multiple Clp targets have now been identified through the combination of molecular genetics, proteomics, and biochemical approaches. For instance, turnover of the thylakoid copper transporter PAA2 is dependent on both ClpC chaperones, as well as ClpPR core complexes, but mechanisms for conditional PAA2 selection for degradation are unknown. Our recent affinity purification study suggested a small set of proteins selected for degradation by ClpS1. The broad range of functions of these ClpS1 targets shows that the Clp protease regulates multiple biological processes; yet the degron(s) responsible for such ClpS-driven substrate selection remain elusive. The existence of ClpS1 in plastids and the conservation of key features in ClpS and its chaperone partner, supports the idea of an N-end rule-like pathway involving plastid Clp system but N-degrons remains to be defined. We believe that identification of degrons for Clp degradation will be essential to truly understand the role of the Clp system in plastid proteostasis. Furthermore, identification of degrons for other plastid proteases will be needed to understand how and to what extent plastid proteases form a functional network. Finally, it is also not clear why the

ClpPR protease core in plastids is not mono-oligomeric such as in plant mitochondria and most non-photosynthetic prokaryotes, and why most ClpR proteins (lacking the catalytic residues) are essential for Clp core function. It will be important to understand if and how ClpPR core diversification, and the presence of the unique ClpT proteins, represents a specific adaptation to the plastid and its proteome. In vivo substrate trapping experiments have been successful in non-photosynthetic bacteria [120,135,169] and the analysis of degrons within such trapped substrates, combined with *in vitro* reconstitution of plastid Clp activities, are likely to provide more definite answers to Clp substrates and significance for chloroplast biology.

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