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Review

The Clp protease system; a central component of the chloroplast protease network ☆

Paul Dominic B. Olinares [1], Jitae Kim, Klaas J. van Wijk *

Department of Plant Biology, Cornell University, Ithaca, NY 14853, USA

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ABSTRACT

Intra-plastid proteases play crucial and diverse roles in the development and maintenance of non-photosynthetic plastids and chloroplasts. Formation and maintenance of a functional thylakoid electron transport chain requires various protease activities, operating in parallel, as well as in series. This review first provides a short, referenced overview of all experimentally identified plastid proteases in *Arabidopsis thaliana*. We then focus on the Clp protease system which constitutes the most abundant and complex soluble protease system in the plastid, consisting of 15 nuclear-encoded members and one plastid-encoded member in *Arabidopsis*. Comparisons to the simpler Clp system in photosynthetic and non-photosynthetic bacteria will be made and the role of Clp proteases in the green algae *Chlamydomonas reinhardtii* will be briefly reviewed. Extensive molecular genetics has shown that the Clp system plays an essential role in *Arabidopsis* chloroplast development in the embryo as well as in leaves. Molecular characterization of the various Clp mutants has elucidated many of the consequences of loss of Clp activities. We summarize and discuss the structural and functional aspects of the Clp machinery, including progress on substrate identification and recognition. Finally, the Clp system will be evaluated in the context of the chloroplast protease network. This article is part of a Special Issue entitled: Regulation of Electron Transport in Chloroplasts.

1. Background

Development and maintenance of chloroplasts requires multiple intra-plastid proteases. The functions of plastid proteases include i) N-terminal processing of thousands of nuclear-encoded precursor proteins, as well as processing of two plastid-encoded precursor proteins (the D1 protein of Photosystem II and cytochrome *f* of the cyt*b6f* complex), ii) removal of unwanted or damaged proteins, such as the D1 protein after light-induced damage, or protochlorophyllide reductase A (PORA) after greening, iii) recycling of amino acids, and iv) control of plastid gene expression. It is important to note that no specific proteases have yet been identified for functions iii) or iv), but there must be proteases that carry out these functions. Table 1 provides an inventory of experimentally identified plastid proteases, their intra-plastid localization and references for experimental identification. Additional chloroplast proteases are predicted based on their putative N-terminal chloroplast targeting sequence but they are not presented or discussed.

One set of identified proteases has demonstrated or suggested roles in cleavage or digestion of precursor sequences used for intracellular targeting of proteins to the plastid (the cTP) or into the thylakoid lumen (lTP) (Table 1). These proteases include the stromal processing peptidase (SPP) involved in precursor processing of most nuclear-encoded, chloroplast-targeted proteins [1], stromal presequence proteases PreP1,2 likely involved in degradation of cleaved transit peptides [2], and the thylakoid processing peptidases TPP [3,4], Plsp1 [5,6] and cTPA [7–9]. Interestingly, Plsp1 is localized in the envelope membranes of developing plastids, but predominantly in thylakoids in chloroplasts [5,10].

There are three larger families of plastid proteases, each of bacterial origin, including the ATP-dependent Zn-metallo protease FtsH family [11,12], the ATP-independent Deg/HtrA family of serine endopeptidases [13–16] and the ATP-dependent serine-type Clp family [12]. The plastid-localized FtsH members are located in the thylakoid or the inner envelope membranes, and in particular the thylakoid members (FtsH1,2,5,8) are well studied [17–19] (Table 1). Loss of these thylakoid FtsH proteins leads to a variegated leaf phenotype with white sectors that contain non-photosynthetic plastids and green sectors containing fully functional chloroplasts [11]. In addition, four FtsH family members that harbor ATP-binding domains but lack the Zn-binding motif for catalytic activity are proposed to be catalytically inactive (designated as FtsHi) and might instead act as chaperones [20]. These FtsHi proteins are predicted to be plastid-targeted using the software TargetP [21] and have been identified in chloroplast proteome analyses in Arabidopsis [22]. Mutants of two of these FtsHi proteins (FtsHi2 and FtSHi3) are embryo lethal (www.seedgenes.org) [23] indicating a crucial role in

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* Corresponding author. Fax: +1 607 255 3664.
E-mail address: kv35@cornell.edu (K.J. van Wijk).
[1] Affiliated with the Graduate Program of the Department of Chemistry and Chemical Biology, Cornell University.

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Table 1
Arabidopsis plastid proteases that have been experimentally verified.[a]

Accession	Annotation	Plastid location[b]	References[c]	PPDB
Protein Processing				
AT5G42390	SPP	S	[1]	+
AT3G19170	Prep1	S + mitochon[d]	[2]	+
AT1G49630	Prep2	S + mitochon[d]	[2]	+
AT3G24590	Plsp1	IES, TL	[5,6]	+
AT2G30440	TPP	TI	[4]	-
AT3G57680	CtpA-1	TL	[8]	-
AT4G17740	CtpA-2	TL	[9]	+
FtsH - Zn-metallo protease family				
AT1G50250	FtsH1	TI	reviewed in [11,12,20]	+
AT2G30950	FtsH2	TI	reviewed in [11,12,20]	+
AT5G42270	FtsH5	TI	reviewed in [11,12,20]	+
AT5G15250	FtsH6	TI	reviewed in [11,12,20]	-
AT3G47060	FtsH7	IEI	reviewed in [11,12,20]	-
AT1G06430	FtsH8	TI	reviewed in [11,12,20]	+
AT5G58870	FtsH9	TI	reviewed in [11,12,20]	+
AT5G53170	FtsH11	IE	reviewed in [11,12,20]	+
AT1G79560	FtsH12	IEI + mitoch[d]	reviewed in [11,12,20]	+
AT4G23940	FtsHi1*	TI	reviewed in [11,12,20]	+
AT3G16290	FtsHi2*	TI	reviewed in [11,12,20]	+
AT3G04340	FtsHi3*	TI	reviewed in [11,12,20]	+
AT5G64580	FtsHi4*	TI	reviewed in [11,12,20]	+
Deg - ATP-independent serine-type protease family				
AT3G27925	DegP1	TL	reviewed in [13]	+
AT4G18370	DegP5	TL	reviewed in [13]	+
AT5G39830	DegP8	TL	reviewed in [13]	+
AT2G47940	DegP2	TS	reviewed in [13]	+
AT3G03380	DegP7	TS	reviewed in [13] and see [15]	-
Clp - ATP-dependent serine-type protease family				
ATCG00670	ClpP1	S	see text for references and details	+
AT1G66670	ClpP3	S	see text for references and details	+
AT5G45390	ClpP4	S	see text for references and details	+
AT1G02560	ClpP5	S	see text for references and details	+
AT1G11750	ClpP6	S	see text for references and details	+
AT1G49970	ClpR1	S	see text for references and details	+
AT1G12410	ClpR2	S	see text for references and details	+
AT1G09130	ClpR3	S	see text for references and details	+
AT4G17040	ClpR4	S	see text for references and details	+
AT5G50920	ClpC1	S, IES	see text for references and details	+
AT3G48870	ClpC2	S	see text for references and details	+
AT5G51070	ClpD	S	see text for references and details	+
AT4G25370	ClpT1	S	see text for references and details	+
AT4G12060	ClpT2	S	see text for references and details	+
AT1G68660	ClpS	S	see text for references and details	+
Other proteases				
AT1G73990	SppA	TI	[26]	+
AT5G35220	EGY1	TI	[25]	+
AT5G05740	EGY2	TI		+
AT2G32480	AraSP	IEI	[28]	+
AT1G09750	CND41	e	[30–32] (tobacco)	+
AT5G25752	Rhomboid protease	IEI	[29]	-
AT3G05790	Lon4 protease	TI + mitoch[d]	[27]	-
AT2G47390	Glutamyl endopeptidase (cGEP)	S	[33,34] (spinach, pea)	+
AT5G65620	Oligopeptidase A	S		+
Aminopeptidases (AP)				
AT1G13270	Methionine AP 1B	S + mitochon[d]	[36]	+
AT3G25740	Methionine AP 1C	S + mitochon[d]	[36]	-
AT4G37040	Methionine AP 1D	S + mitochon[d]	[36]	+
AT4G30920	Leucyl AP 2	S	[35]	+
AT4G30910	Leucyl AP 3	S	[35]	+
AT3G05350	APP2 (M24 peptidase type)	S	[35]	+
AT1G63770	Glycyl AP	S	[35]	+

[a] Includes proteases that are not yet characterized in Arabidopsis but are homologous to those found in other plant or bacterial species and have been identified in multiple, independent mass spectrometry-based chloroplast proteomics experiments in Arabidopsis (see Plant Proteome Database, PPDB).
[b] Subplastidial location: IEI - inner envelope membrane, integral-bound; IES - inner envelope membrane-associated, stroma side; S - stroma; N- nucleoid (protein-DNA complexes in plastids); TS - thylakoid membrane-associated, stroma side; TI - thylakoid membrane, integral-bound; TL - thylakoid membrane-associated, lumenal side; L - lumen.
[c] The plant species other than Arabidopsis where the protease homologue is characterized is indicated in parenthesis.
[d] Dually targeted to chloroplasts and mitochondria.
[e] These FtsH homologues are presumed catalytically inactive because they lack the conserved Zn-binding site.

chloroplast development, warranting further analysis. The Deg proteins are soluble ATP-independent serine-type proteases and the plastid contains both lumenal (Deg1,5,8) and stromal members (Deg2,7); several of the Deg proteins play a role in Photosystem II assembly and/or the damage-repair cycle [13–16,24]. The Clp family members are localized in the stroma with some association to the chloroplast membranes, in particular the ClpC chaperones which interact with the inner envelope protein translocation complex (TIC)

[12,18]. Additional plastid proteases [25–34] have been studied and several soluble aminopeptidases [35,36] have been identified (Table 1). A recent review summarizes existing knowledge for most of these identified plastid proteases [19]. We have also included other peptidases and proteases in Table 1 that have not yet been characterized in *Arabidopsis* but have been identified in proteomic analyses of chloroplasts [22] (see also the Plant Proteome Database, PPDB at http://ppdb.tc.cornell.edu/). They have been assigned putative functions based on homology to known proteases, but we will not further discuss them here.

2. The Clp protease machinery

2.1. Overview of the Clp system

In this review, we focus on the role of the abundant Clp protease system in plastids. A number of studies have shown the functional importance of the Clp system in *Arabidopsis thaliana*, tobacco and the green algae *Chlamydomonas reinhardtii*. Since publication of the last (brief) review on the plastid Clp protease system [12], significant progress has been made in this research area, thus providing motivation and justification for a new review on the plastid Clp system. Furthermore, numerous studies have been published in the last few years on the molecular, structural and functional analysis of the Clp system in non-photosynthetic bacteria (in particular *Escherichia coli* and *Bacillus subtilis*), as well as photosynthetic bacteria (*Synechococcus*). Therefore, this review will also cover aspects of the bacterial Clp system that are relevant to the plant plastid Clp system. Finally, we will discuss what is known about Clp substrates and substrate selection in plastids as well as situate the Clp system within the chloroplast protease network.

The Clp (caseinolytic protease) family has been found in almost all bacterial species and eukaryotic organelles except for archaea, mollicutes and some fungi [37]. The Clp machinery has two oligomeric components, namely i) a barrel-shaped tetradecameric protease core with the catalytic sites sequestered inside the complex and ii) hexameric ring-like ATP-dependent chaperones. The chaperones recognize specific substrates with or without the aid of adaptors, unfold these substrates and translocate them into the proteolytic core for degradation [38,39]. Compartmentalization of the proteolytic sites within the core complex and coupling with chaperones and associated factors for substrate delivery enable targeted, adaptive and regulated protein degradation within the cell.

The Clp machinery is well studied in the Gram negative bacterium *Escherichia coli*, where it was first isolated and characterized [40,41]. The *E. coli* ClpP peptidase core is a homotetradecameric complex consisting of two stacked heptameric rings with the active sites enclosed within the equatorial cavity [42]. Access to this cavity is restricted by narrow axial entrance pores [42]. By itself, the Clp core can slowly degrade small peptides but requires the cooperation of ATP-driven chaperones to digest larger peptides or denatured proteins [43–45]. These chaperones include ClpX and ClpA, which harbor one or two AAA+ (ATPase associated with cellular activities) domains, respectively. They form hexamers that mount coaxially on the ClpP core for substrate delivery [44]. The interaction between the axial surfaces of the Clp core with the chaperone rings is highly dynamic, partly due to the symmetry mismatch between the hexameric chaperone and (double) heptameric protease core [46–48]. Substrates are fed axially into ClpP [49,50] and degraded processively into peptides of seven to eight residues [51]. In addition, the association of a small adaptor protein, ClpS, confers additional substrate specificity to ClpAP complexes. ClpS changes the affinity of ClpA towards protein aggregates [52] and modulates the recognition and delivery of substrates with N-terminal degradation signals known as N-end rule substrates [53–55]. ClpS binds to the N-domain of ClpA [56,57]. A molecule of ClpS can associate with high affinity to ClpA hexameric rings and is sufficient to induce ClpS-mediated substrate delivery and degradation by the ClpAP complex [58]. The Gram positive bacterium *B. subtilis* is a free living bacterium and is much better adapted to higher temperatures than *E. coli*; these differences in temperature tolerance are likely to affect the protein homeostasis machineries, including proteases [59]. *B. subtilis* has three Clp chaperones (ClpC, ClpE and ClpX) and one ClpP; surprisingly it lacks the ClpP-independent disaggregase ClpB which is present in *E. coli* and higher plant plastids. Unlike most other organisms, ClpP in *B. subtilis* is monomeric *in vivo* (and *in vitro*) but oligomerizes upon interaction with its adaptor-activated Clp chaperones, in particular MecA [60,61]. The ClpC chaperones depend on different adaptors such as MecA, YpbH, MscB, ClpS, whereas ClpX interacts with the adaptor YjbH [59]. Interestingly, non-photosynthetic plastids (apicoplasts) of the human malaria parasite *Plasmodium falciparum* contain both a ClpP and a ClpR protein [62]. Both ClpP and ClpR form mostly homoheptameric rings as observed by size-exclusion chromatography, analytical ultracentrifugation and electron microscopy. The X-ray structure of *Plasmodium* ClpP showed the protein complex as a compacted tetradecamer similar to that observed for *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* ClpPs [62].

The Clp machinery in the photosynthetic bacterium *Synechococcus* sp. PCC 7942 consists of three ClpP genes (P1,2,3), one ClpR, ClpX and ClpC gene, as well as two ClpS genes [63]. The ClpR protein is structurally similar to ClpP but lacks the catalytic residues for peptide bond hydrolysis. It is proteolytically inactive but its presence in the Clp core appeared not to limit the overall proteolytic activity [64]. The cyanobacterial ClpC is a ClpA orthologue and exhibits protein refolding and protein disaggregation activities [65]. Cyanobacteria possess two different functional Clp assemblies, namely a dispensable ClpP1,2 complex associating with ClpX and an essential ClpP3/ClpR complex interacting with ClpC [63]. In addition, the two adaptors ClpS1 and ClpS2 associate only with ClpC and not with ClpX [63]. In another cyanobacterial species, *Nostoc* sp.PCC7120, ClpC has been shown to interact with NblA, a protein involved in the degradation of the light-harvesting phycobilisome complexes [66]. This interaction is ATP/ADP-dependent and NlbA is proposed to act as an adaptor for ClpC towards Clp-mediated phycobilisome clearance [66].

In the green alga *C. reinhardtii*, three ClpP genes (*CLPP1, CLPP4, CLPP5)* and five ClpR genes (*CLPR1-CLPR4, CLPR6*) encode for the chloroplast-localized hetero-oligomeric Clp protease core complex [67]. Interestingly, plastid-encoded *clpP1* contains a large insertion sequence (IS1) and several ClpP1 protein variants have been observed within the Clp core, including the precursor $ClpP_{1H}$ and several processed versions which lack various segments within the IS1 [67,68]. The spliced forms do not arise from mRNA splicing [69], nor from protein intron self-splicing as in inteins, but instead from endoproteolytic cleavage within the IS1 region of $ClpP1_H$, mostly likely after the assembly of the $ClpP1_H$-containing protease complex [68].

The plastid-localized Clp proteolytic system further diversified in higher plants. The dicotyledon *A. thaliana* has five serine-type Clp proteases (P1, P3-P6), four non-proteolytic ClpRs (R1–4), three Clp AAA+ chaperones (C1, C2, D) similar to the *E. coli* ClpA, the adapter ClpS (homologous to the *E. coli* ClpS) and ClpT1, T2 with unknown functions but with similarity to the N-terminal domain of bacterial ClpA [12,70]. ClpB3, another plastid-localized AAA+ chaperone, lacks the I(L)GF motif combined with an upstream basic residue [70] which is implicated in interaction with the Clp protease core [71]. Therefore, ClpB3 is unlikely to directly associate with the plastid Clp core complex. We identified all Clp proteins by mass spectrometry in chloroplasts of *Arabidopsis* and non-green plastids in *Brassica rapa* roots and *Brassica oleracea* petals [70,72,73]. We note that mass spectrometry-based identification of the relatively small 12 kDa ClpS protein was challenging and was achieved only after size exclusion fractionation of stroma [73] (also, see the Plant Proteome Database, PPDB at http://ppdb.tc.cornell.edu/). Published transcript analysis

suggested that the *CLP-PRC* genes are constitutively expressed in plants, with only minor changes in gene expression under specific stress conditions or during senescence [74–76]. Based on publicly available microarray data sets of various organs and their developmental state in Atgenexpress (http://arabidopsis.org/), and the current (more complete) information of the Clp system, we reevaluated this conclusion (Fig. 1). We first evaluated the individual nuclear-encoded plastid Clp members, except for *CLPC2* since it was not represented in the data set. This showed that the 8 *CLPP/R* genes all co-expressed quite tightly. Similar co-expression was observed from *CLPT1,2*. Therefore, we calculated the median accumulation levels for these two gene groups. Fig. 1 shows the mRNA levels for roots, cotyledons, a developmental series of leaves (from a 17 day old plant), senescing leaves, various flower organs and a series of developing seeds that mostly reflects transcripts in the developing embryos. *CLPPR* and in particular *CLPT* transcript levels increased during leaf development and aging, whereas transcript level for *CLPS, D* and *C1* decreased (Note: ClpS and ClpC1 also decrease with increasing leaf age at the protein level; unpublished data Asakura and van Wijk). Interestingly, *CLPT* transcript levels were strongly reduced (3- to 4-fold) during leaf senescence. In contrast, *CLPD* expression was strongly induced during senescence as shown in previous studies [75,77,78], however ClpD protein levels did not increase, but instead decreased, suggesting post-transcriptional control [78]. The *CLP* transcripts were also found in various floral organs but not in mature pollen. *CLP* genes were expressed in the developing embryo (seed) and their transcript abundance remained similar during embryogenesis from torpedo to cotyledon stage, with the exception of *CLPD* (Fig. 1). *CLPT1,2* and *CLPS* transcript levels generally followed *CLP-PRC*, with low expression in roots, virtually no expression in pollen and high expression levels in cotyledons, leaves and most components of the influorescence (data not shown). Collectively, these transcript data indicate that the Clp protease family is particularly important in earlier stages of chloroplast development. *CLPS* co-expressed with *CLPC* and *CLPPR*, whereas the *CLPT1,2* genes also generally co-expressed with *CLPPR* family, except that the *CLPT1,2* genes appeared to increase importance with maturation of chloroplasts, but decreased during senescence, suggesting that they play a specific role in chloroplast maintenance. Quantitative proteome analysis of the developing maize leaf showed that the Clp machinery has the highest abundance during the transition of proplastids to chloroplasts [79]. Moreover, the Clp complex was observed as one of the prominent complexes in pea etioplasts suggesting an important role in the biogenesis of etioplasts and their transition into chloroplasts [80]. Thus in general Clp protein accumulation patterns in leaves correspond with mRNA expression levels.

ClpR1, R3 and R4 have a 10-12 amino-acid insertion domain compared to the ClpP1-6 proteins, ClpR2 and *E. coli* ClpP [70]. Our homology models suggested that this domain is protruding into the tunnel of the ClpP/R core, possibly affecting substrate presentation to the catalytic sites [70]. Additional distinct features of chloroplast ClpR and ClpP proteins compared to the *E. coli* ClpP are the extended C-termini, that might influence protein interactions to the adaxial site(s) of the ClpPR core, i.e. with hexameric rings of ClpC,D or with ClpT1,T2 [70].

Surprisingly, our analyses of the native soluble proteome of non-green plastids in roots and petals of *Brassica rapa* and *Brassica oleracea,* respectively, as well as chloroplasts of *A. thaliana,* have shown that they all contain a stromal 325–350 kDa Clp core protease complex consisting of all the nine Clp proteins (ClpP1, P3-P6, R1-R4)

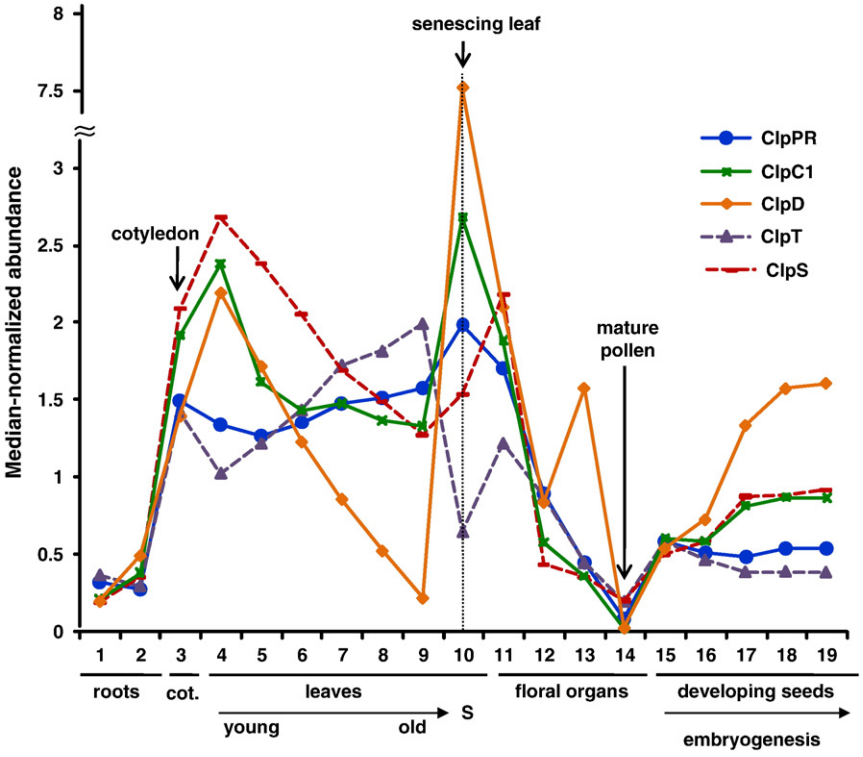

Fig. 1. The gene expression levels of the plastid-targeted Clp proteins (except ClpP1 and ClpC2) in *Arabidopsis* from various plant organs and at different developmental stages [167]. Normalized transcript abundances are shown for the root at 7 days (1) and 21 days (2); cotyledons (3); a 17-day old rosette with leaf #2 (4), #4 (5), #6 (6), #8 (7), #10 (8) and #12 (9); a senescing leaf at 35 days of growth (10); floral organs isolated at stage 12 with 21+ days including sepals (11), petals (12) and stamen (13); mature pollen (14); seeds taken after 8 weeks and after silique removal with mid to late torpedo embryos (15), late-torpedo to early walking-stick embryos (16), walking stick to early curled cotyledons (17), early curled to early green cotyledons (18) and green cotyledon embryos (19). Gene microarray data was obtained from AtgenExpress (http://jsp.weigelworld.org/expviz/expviz.jsp). mRNA abundances were normalized to the median abundance level for each gene across all samples. The median-normalized abundances for ClpP3/4/5/6 and ClpR1/2/3/4 were similar and were averaged into ClpPR. Likewise, the abundances for ClpT1 and T2 were averaged as ClpT.

and ClpT1 and T2 [70]. Furthermore, separation of these complexes by native isoelectric focusing showed a single complex in each plastid type (at pI of ~5) [70]. Native gel electrophoresis of the ClpPR complex combined with Western blot analysis suggested the existence of two Clp core subcomplexes (heptameric rings): a ~180–200 kDa ring containing ClpPs (ClpP3-P6) and a ~230 kDa ring containing all the ClpRs (R1-R4) and ClpP1 [81]. Furthermore, this western blot analysis also suggested that ClpT1 binds to the ClpP3–P6 ring [81]. We point out that the individual rings have not yet been purified and that the precise stoichiometry within the rings is not clear, even if tentative quantification was obtained from gel band stain intensities for Clp proteins from stromal proteome analyses [70,82]. In contrast to plastids, plant mitochondria contain a single homotetradecameric ClpP2 complex, presumably associated with ClpX chaperones [70,83].

ClpT1,2 are unique to land plants and have not been found in algae or (cyano)bacteria [72]. From our threading and structural modeling analysis, it was concluded that ClpT1 and T2 cannot fit within the Clp core ring structure but they dock well on the Clp core's aromatic pockets situated near the axial entrance [70]. From these modeling studies, we proposed several possible roles for ClpT. For instance, ClpT may regulate the association of the hexameric ClpC1,C2 or D with the core by competing for docking sites on the core. Based on homology to the *E. coli* ClpA, ClpT1,2 have predicted binding sites for interactions with the adaptor ClpS [56,70]. As such, plastid ClpT1,2 might provide docking sites for ClpS to deliver substrates, most likely short peptides, into the core.

The Clp chaperones ClpC1 and C2 were found as dimers at ~200 kDa and after interaction with their substrates they are predicted to dock as hexameric rings on either axial side of the proteolytic core [70,84,85]. However, the Clp core associated with the ClpC or D chaperone complex has not yet been isolated; however this will be important to i) more firmly establish ClpC/D function, ii) to determine if ClpC/D can bind to both adaxial sites of the ClpPR core, and iii) to understand its relationship with ClpT1,2 (i.e., whether ClpC/D can associate with a ClpPRT complex). Finally, little is known about plastid ClpS. It remains to be determined if ClpS does interact with the ClpC/D chaperones or ClpT1,2 and the adaptor function of ClpS has not been demonstrated in plants.

2.2. Lessons from Clp gene disruption studies in photosynthetic organisms

Arabidopsis mutants with reduced or complete loss of expression of nuclear-encoded Clp genes have been generated by anti-sense RNA, by T-DNA insertions and by EMS mutagenesis. Table 2 provides an overview of published Clp mutants in *Arabidopsis*. Phenotypes for mutant lines with visible phenotypes (yellow-pale green and/or seedling lethal) in *Arabidopsis* are shown in Fig. 2 (Documented embryo lethal mutants for *CLPP4,5* and *CLPC1xC2*–see Table 2–are obviously not shown). So far, mutants for three of the four ClpR proteins and all four nuclear-encoded plastid-localized ClpP proteins (P3–6) have been obtained and analyzed in varying degrees. Down-regulation of the nuclear-encoded *CLPR2* gene by 80% delayed chloroplast biogenesis and affected protein homeostasis [86,87]. Similarly, partial down-regulation of *ClpP4* and of *ClpP6* by anti-sense RNA techniques reduced plant growth and development and resulted in pale-green plants [81,88]. Reduced levels of ClpR1 protein

Table 2
Arabidopsis *CLP* mutant lines.

Gene	Locus	Mutant Name	Phenotype	Germplasm	Source	Ecotype	Position of the T-DNA insertion or mutation	Refs
CLPP3	At1g66670	***clpp3-1***	Seedling Lethal	SALK_065330	SALK	Col-0	3/5 Exon	[92]
CLPP4	At5g45390	***clpp4 antisense***	Variegated	clpp4 antisense	B. Zheng	Col-0	antisense	[88]
		clpp4-1	Embryo Lethal	SALK_000913	SALK	Col-0	5' UTR (−16)	[92]
CLPP5	At1g02560	***clpp5-1***	Embryo Lethal	SALK_007708	SALK	Col-0	4/9 Exon	[90]
		clpp5-2	Embryo Lethal	SAIL_59_E07	Syngenta	Col-3	5/8 Intron	[90]
		clpp5-3	Embryo Lethal	SAIL_876_G06	Syngenta	Col-0	2/9 Exon	[90]
CLPP6	At1g11750	***clpp6 antisense***	Pale Green, Yellow	clpp6 antisense	L. Sjögren	Col-0	antisense	[81]
CLPR1	At1g49970	***clpr1-1***	Pale Green, Yellow	clpr1-1, EMS	S. Koussevitzky	Col-0	7/9 Exon	[89,90]
		clpr1-2	Pale Green, Yellow	SALK_088407	SALK	Col-0	1/8 Intron	[89,90]
CLPR2	At1g12410	***clpr2-1***	Pale Green, Yellow	SALK_046378	SALK	Col-0	5' UTR (−7)	[86,90]
		clpr2-2	Seedling Lethal	SALK_016774	SALK	Col-0	2/9 Exon	[90]
		clpr2-3	Seedling Lethal	SAIL_98_C01	Syngenta	Col-0	6/8 Intron	[90]
CLPR4	At4g17040	***clpr4-1***	Seedling Lethal	JP7_7H07L	J. Ecker	Col-0	2/6 Intron	[90]
		clpr4-2	Seedling Lethal	GK-692C06	GABI-Kat	Col-0	1/7 Exon	[90]
CLPT1	At4g25370	***clpt1-1***	Wild-Type Like	SALK_052772	SALK	Col-0	3/5 Intron	
		clpt1-2	Wild-Type Like	GK-285A06	GABI-Kat	Col-0	5/6 Exon	
CLPT2	At4g12060	***clpt2-1***	Wild-Type Like	SAIL_340A10	Syngenta	Col-0	3/5 Exon	
		clpt2-2	Wild-Type Like	SALK_132943	SALK	Col-0	4/4 Intron	
CLPS	At1g68660	***clps***	Wild-Type Like	SAIL_326B_G12	Syngenta	Col-0	2/3 Exon	
CLPC1	At5g50920	***clpc1-1 = hsp93-V-2***	Pale Green, Yellow	SALK_014058	SALK	Col-0	4/9 Exon	[93,95]
		clpc1-2 = hsp93-V-1	Pale Green, Yellow	SAIL_873_G11	Syngenta	Col-0	7/9 Exon	[93,94,95]
		clpc1-3 = irm1	Pale Green, Yellow	irm1	H. Wu	Col-0	9/9 Exon	[99]
CLPC2	At3g48870	***clpc2-1***	Wild-Type Like	clpc2-1, EMS	S. Park	Ler	8/8 Intron	[162]
		clpc2-2 = hsp93-III-1	Wild-Type Like	SAIL_622_B05	Syngenta	Col-0	9/9 Exon	[99]
		clpc2-3 = hsp93-III-2	Wild-Type Like	GK-039E12	GABI-Kat	Col-0	7/9 Exon	[99]
CLPD	At5g51070	***clpd***	Wild-Type Like	SAIL_77_G05	Syngenta	Col-0	1/12 Exon	
CLPB3	At5g15450	***clpb3-1 = clpB-p2***	Pale Green, Yellow	SALK_111575	SALK	Col-0	1/9 Exon	[168,87]
		clpb3-2 = clpB-p1 = apg6-3	Pale Green, Yellow	SALK_071039	SALK	Col-0	4/8 Intron	[123,168]
		clpb3-3 = apg6-1	Pale Green, Yellow	apg6-1, Ds	F. Myouga	No-0	5'UTR	[123]
		clpb3-4 = apg6-2	Pale Green, Yellow	apg6-2, Ds	F. Myouga	No-0	2/9 Exon	[123]
		clpr1-2 × clpr2-1	Seedling Lethal					[90]
		clpb3-1 × clpr2-1	Seedling Lethal					[87]
		clpc1-2 × clpc2-2	Pale Green, Yellow, Smaller. (Stronger mutant than *clpc1-2*)					[96]
		clpc1-2 × clpc2-3	Embryo Lethal					[96]
		clpc2-1 × var2 (ftsh2)	*clpc2-1* suppresses variegated phenotype					[162]
		clpc1 × var2 (ftsh2)	*clpc1* suppresses variegated phenotype					[127]
		clpr1 × var2 (ftsh2)	*clpr1* suppresses variegated phenotype					[127]

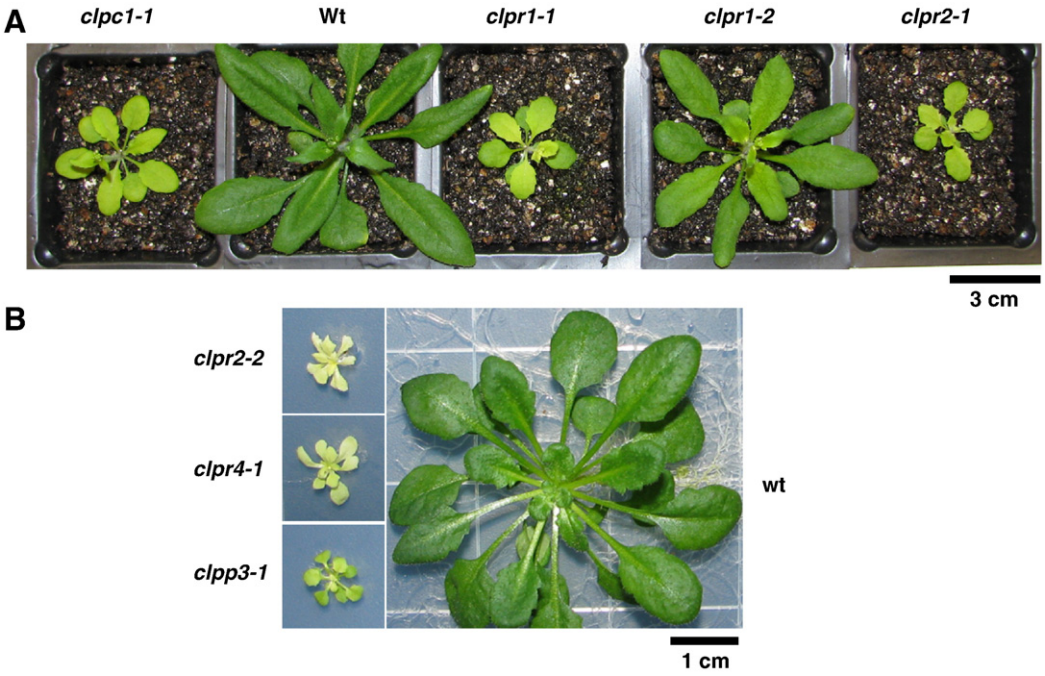

Fig. 2. Growth and Development of wild-type and the different Clp mutants. A. Direct comparison of wild-type, *clpc1-1*, *clpr1-1*, *clpr1-2*, and *clpr2-1* mutants grown on soil for 28 days under a 16/8 hour light/dark cycle at 120 μmol photons m^{-2} s^{-1}. Bar = 3 cm. More information about these mutants is provided in Table 2. B. Homozygous *clpr2-2*, *clpr4-1*, and *clpp3-1* plants on MS agar plates with 2% sucrose grown for 2 months under a 10/14 hour light/dark cycle at 40 μmol photons m^{-2} s^{-1}. Bar = 1 cm. More information about these mutants is provided in Table 2.

by EMS mutagenesis (*clpr1-1*) or T-DNA insertion (*clpr1-2*) to nearly undetectable levels resulted in virescent mutants, but *clpr1-1* plants did grow on soil and set viable seeds without problem [89,90]. In case of null mutants for *CLPR2* and *CLPR4*, the seeds did germinate and under heterotrophic conditions, pale green seedlings developed slowly but produced no viable seeds [90]. The relatively mild phenotype of the *CLPR1* mutants could be explained by a partial functional substitution by ClpR3 [90,91]. Null mutants in *CLPP4* and *CLPP5* were blocked in embryogenesis and never germinated, even when supplied with sugars [90,92]. Interestingly, null mutants in *CLPP3* are not embryo lethal, but are seedling lethal on soil; however they can be rescued under heterotrophic conditions [92]. Collectively, analyses of Clp mutants revealed that the ClpPR core subunits, except for ClpR1, exhibit little functional redundancy and that the Clp protease core is essential for plastid development both during embryogenesis and in cotyledons and leaves.

Mutants for ClpC1,C2 and D, as well as ClpB3, have been analyzed in fair detail (see Table 2 for references and other details). Loss of expression of the *ClpC1* chaperone resulted in reduced plant growth and chloroplast development, but homozygous plants remained autotrophic, producing viable seeds [93–95]. Mutants lacking both ClpC1 and ClpC2 however were blocked in embryogenesis [96]. Although ClpC1,2 accumulate predominantly in the stroma, ClpC1 is also associated with the chloroplast protein translocation machinery in the inner envelope, interacting in particular with Tic110 and Tic40 [97,98]. Consistently, loss of ClpC1 results into lower protein import rates into isolated chloroplasts [94,95]. Recently it was reported that the visible pale-green phenotype of a *CLPC1* mutant (*irm1*) harboring a point mutation (Gly773 into Arg773) can be complemented by providing extra iron to the plants, but not by extra zinc or manganese, suggesting altered chloroplast import of nuclear-encoded proteins involved in iron transport in *irm1* [99]. Further characterization of this interesting phenomenon is needed.

Whereas the most comprehensive genetic analysis of nuclear-encoded Clp genes was performed in *Arabidopsis*, down-regulation of the plastid-encoded *CLPP1* gene was done in tobacco, which showed that the ClpP1 protein is essential for shoot development [69,100]. Down-regulation of the *CLPP1 gene* in the green algae *C. reinhardtii* suggested that ClpP1 is involved in the degradation of the thylakoid proteins, in particular in genetic backgrounds that lead to miss-assembly of thylakoid complexes [101,102]. Furthermore, in the photosynthetic bacterium *Synechococcus* sp. PCC 7942, mutant analyses have revealed that *CLPP and CLPP2* are dispensable but that *CLPP3* and *CLPR* are essential to cell viability [63,103,104].

2.3. Large scale leaf protein quantification of wt and Clp mutants seedlings by spectral counting

To better understand the role of the Clp protease machinery in chloroplast biogenesis and protein homeostasis and to discover potential Clp substrates, comprehensive proteome analyses of Clp mutants can be highly informative. Such a systems approach would also provide insights into the plastid protein homeostasis network (i.e., chaperones, isomerases, plastid gene expression, protein import, proteases) and the place of the Clp protease system within this network. Recent improvements in sensitivity, mass accuracy and speed of mass spectrometers [105,106] have enabled large-scale proteome quantifications with a better coverage and higher sensitivity than two-dimensional (2DE) gel image-based quantification methods. We took advantage of these new developments, and using a fast and accurate mass spectrometry (MS) instrument (LTQ-Orbitrap) [107,108], we performed comparative proteome analysis of unfractionated leaf proteomes from *clpr2-1* and *clpr4-1* mutant seedlings [87,90] employing a label-free MS-based quantification technique known as spectral counting [22,109–112]. Although it might be possible to isolate intact chloroplasts from young seedlings of Clp mutants with strong phenotypes, it is far more practical if one can assess the chloroplast proteome without actual isolation, with the added benefit that the cellular response outside the chloroplast can also be evaluated and the selection of plastids with specific characteristics can be avoided. Analysis at the leaf proteome level is also made feasible because the relatively abundant 1200–1300 chloroplast proteins have now been identified in *Arabidopsis* from a large number of (independent) chloroplast proteomics studies,

supplemented by many detailed experimental papers for individual plastid-localized proteins. Assembly of these proteomics studies, followed by manual evaluation, annotation and deposition in public databases such as the Plant Proteome Database, PPDB (http://ppdb.tc.cornell.edu/), have enabled efficient analyses of chloroplast-localized proteins from large-scale leaf proteome datasets.

We analyzed the leaf proteomes of the soil-grown *clpr2-1* mutant at different time points of leaf development (stages 1.07 and 1.14) [87] and that of the *clpr4-1* null mutant grown under heterotrophic conditions (on MS plates with sucrose) [90]. Since these mutants were delayed in development, comparisons with wild-type were made between identical growth stages rather than identical plant age since comparison by developmental stage yields the most biological relevance (see for more on this topic in [113]). From both large-scale experiments, we identified more than 2800 proteins and quantified ~700 of them (reliable quantification of a protein requires that the number of matched spectra passes a minimal threshold as determined by statistical analysis (see [87,90] for discussion), providing a comprehensive dataset for further analysis. The seedling proteome analysis showed that the strongest effects occurred within the chloroplast. Plastid biogenesis and protein homeostasis are functionally intertwined with primary and secondary metabolism. This connectivity must be accounted for when interpreting the comparative proteomics data if one aims to understand the substrates and the general role of the Clp protease system. In addition, while overaccumulation of a protein in the protease mutants could indicate that it is a substrate for proteolysis, such increase could also represent an indirect, compensatory response due to the mutation. In subsequent sections, we describe the proteins that exhibited significant differential accumulation in the *clpr2-1* and *clpr4-1* mutants from these spectral count studies, as well as a previous *clpr2-1* analysis [86], with the aforementioned caveats in mind. We also comment on the comparative gel analysis of chloroplast proteomes from a *CLPP6* antisense line [81], as well as the *clpr1-1* mutant [91].

2.4. The role of the Clp system in photosynthesis and metabolism

The total thylakoid proteome was reduced at least two-fold in young *clpr2-1* plants [86,87], in particular the lumenal oxygen-evolving complex (OEC) proteins [87]. In contrast, western blot analysis of representative proteins for each photosystem in mature *clpr2-1* plants revealed only slight down-regulation of these proteins [86]. Likewise, several proteins of the photosynthetic complexes and the Rubisco complex were also reduced in more virescent younger leaves but remained similar to wild-type in the older, outer leaves for the rosettes of *clpr1*-1 [91] and the *CLPP6* antisense mutant [81]. The most pronounced phenotype in younger leaves as compared to older leaves on the same rosette plant is consistent with reduced visible phenotypes in older plants of *clpr2-1,* as well as all other *CLPP/R* mutants listed in Table **2**. It is also consistent with the mRNA expression data (Fig. 1). Thus the Clp protease core is less critical for maintenance of the thylakoid proteome than it is for its biogenesis. Alternatively, the maintenance function of the Clp protease system can also be fulfilled by increased activity of other chloroplast proteases, such as the FtsH system. Exhibiting a more severe phenotype than *clpr2-1* and *clpr1-1*, the albino *clpr4-1* showed a major loss of photosynthetic capacity with a strong reduction of the thylakoid-bound photosynthetic apparatus proteins, as well as the soluble Calvin cycle enzymes, in particular the Rubisco complex [90]. Surprisingly, thylakoids of *clpr2-1* accumulated low levels of several unprocessed chlorophyll a/b binding proteins of PSII (LHCII-1,2,3,4,5 and LHCII-3) and PsaF, an integral membrane protein of PSI with a cleavable lumenal transit peptide [86]. An unprocessed Lhcb2 was also observed in *clpr1-1*[89].

Whereas the thylakoid membrane system was clearly reduced as observed by the proteome and microcopy analyses, thylakoid-associated plastoglobules visibly increased in both knockdown (*clpr2-1*) and null (*clpr4-1*) Clp mutants [86,90] indicative of a thylakoid membrane homeostasis problem. Plastoglobules (PG) are thylakoid-associated lipoprotein particles which play a role in quinone, tocopherol and carotenoid metabolism and storage, as well as in stress defense and chlorophyll and thylakoid membrane turnover [114]. The increase in plastoglobule size and number was further reflected in the strong increase in several specific PG proteins, in particular the structural fibrillin FIB1A [87,90].

We also observed upregulation of several plastid enzymes and envelope transporters involved in metabolism. The response of these metabolic pathways can in part be explained by the loss of photosynthetic capacity in Clp mutants. A clear example is the multi-fold upregulation of the inner envelop ATP/ADP translocator which imports cytosolic ATP into the chloroplast [86,87]. Other effects, such as the increase of several enzymes in the 2-C-methyl-D-erythritol-4-phosphate/1-deoxy-D-xylulose-5-phosphate pathway (MEP) pathway producing the isoprene units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) needed for synthesis of e.g. chlorophylls, quinones, tocopherols, carotenoids, can be explained by a combination of developmental effects on gene expression and the reduced electron transport affecting the availability of electrons for reduction steps in the MEP pathway [87]. We did not observe any significant down-regulation of metabolic pathways other than photosynthesis and based on our phenotypic, chemical complementation and proteome analysis, we can exclude several pathways and processes as direct targets for Clp proteolysis and being the direct cause of the albino or embryo lethal phenotype. For instance, feeding experiment with IPP and DMAPP did not affect any of the *clppr* phenotypes, thus suggesting that loss of the MVA or MEP pathway is not the cause of the *clpr1, clpr2* or *clpr4* phenotypes. Moreover, the presence of chlorophyll *a* and *b*, as well as carotenoids, shows that none of these pathways are blocked. Plastids also synthesize several essential vitamins, such as thiamine (vitamin B1) and ascorbate (vitamin C) [115], but supplementation with a vitamin mix, or with thiamine hydrochloride alone, did not complement any of our mutants. Growth of *clpr2-1* or *clpr4-1* alleles under continuous low light, instead of a light–dark cycle, did not affect the *clpr2-1* or *clpr4-1* phenotype, indicating that accumulation of chlorophyll intermediates (which are photosensitizers) in the dark, as observed in the *flu* mutant [116], did not cause the pale green or white phenotype.

2.5. Clp mutants show an increased need for protein import, folding and unfolding capacity

Consistent upregulation of proteins involved in import (Tic110), folding and maturation (Cpn60/cpHSP70/cpHSP90 and ROC4) and unfolding (ClpB3) were observed from our large-scale comparative proteome studies [87,90]. The aforementioned folding and maturation proteins also exhibited differential accumulation in *clpr1-1* [91]. In addition, immunoblot analyses of total soluble leaf proteomes revealed that both ClpC1 and ClpC2 were upregulated in *clpr2-1* [86]. CPN60α,ß (GroEL homologues) and cpHSP70-1,2 (DnaK homologues) are the chloroplast chaperones that provide the central folding activity in the chloroplast [117]. HSP90 proteins are typically involved in the late folding steps of proteins [118] and an *Arabidopsis* cpHSP90 mutant, *cr88,* was delayed in chloroplast development in cotyledons and young leaves [119]. ROC4 is a very abundant stromal peptidylprolyl isomerase [22,85] with *in vitro* rotamase activity [120]. Recently two additional functions for ROC4 were suggested, namely the folding of stromal serine acetyltransferase, thereby enabling the cysteine-based thiol biosynthesis pathway to adjust to light and stress conditions [121] and repair of damaged Photosystem II [122]. ClpB3 is the chloroplast homologue of the bacterial ClpB protein [123], which unfolds aggregated proteins aided by the DnaK chaperone system (homologous to cpHSP70) [124,125]. Disaggregation by the bacterial ClpB3 homologue, followed by protein refolding, is critical for cell viability in *E. coli* [126].

The strong upregulation of ClpB3 in both young and mature *clpr2-1* mutants suggests protein aggregation in the chloroplast and a persistent protein folding stress. The *clpb3 x clpr2-1* double mutant is seedling lethal [87] further supporting our observation of perturbed proteome homeostasis in the *clpr2-1* background. Our working hypothesis is that reduced Clp protease activity leads to accumulation of unwanted/damaged proteins that accumulate as protein aggregates; ClpB3, together with cpHSP70, are upregulated in an effort to unfold and reactivate these aggregates. The engagement of cpHsp70 in refolding activity of aggregated plastid proteins likely reduces the capacity for folding of newly imported nuclear-encoded proteins or newly synthesized chloroplast-encoded proteins and thus further contributes to destabilized proteome homeostasis. The upregulation of both CPN60 and cpHSP90 suggests an extra demand for ATP-dependent folding activity, possibly due to less favorable/efficient folding conditions, for instance a limited availability of ATP or due to prolonged engagement of these chaperones with unwanted proteins. The increase in Tic110 suggests a bottleneck in protein import into the chloroplast; we speculate that this is due to delayed release from the import channel and from Tic110, since chaperones including ClpC1, are engaged in unproductive stabilization of proteins, rather than aiding in completion of the import process. This scenario would be consistent for the explanation of the iron deficiency in the *CLPC1* mutant from [99], as discussed above. During and after import of nuclear-encoded proteins, chaperones are also possibly needed for presentation of imported precursor proteins to the stromal processing peptidase (SPP). However, since these chaperones are engaged with aggregates, a subfraction of the imported thylakoid-localized proteins remains unprocessed and is eventually incorporated to the thylakoid membranes, as observed in the *clpr2-1* mutant.

2.6. The role of Clp in chloroplast gene expression

Another molecular consequence of reduced ClpPR activity is delayed 70S plastid ribosome maturation, which presumably affects plastid gene expression and consequently the signaling pathway to the nucleus (see Section 2.9 below). It has been shown that *CLPR1* and *CLPR2* mutants have delayed processing of the dicistronic 23S–4.5S chloroplast rRNA [87,89,127]. Since rRNA processing is tightly coupled to the assembly process, the delayed rRNA processing could either be the cause or the consequence of delayed ribosome assembly. Furthermore, we observed a very strong increase in the RH3 DEAD box RNA helicase and the exoribonuclease polynucleotide phosphorylase, named RIF10 or PNPase. The RH3 DEAD box RNA helicase has not been well-studied in plants, but members of this helicase family are often involved in ribosome maturation and/or rRNA processing and stability [128,129]. RH3 was found migrating with megadalton-sized protein complexes in native stromal fractionations suggesting association with ribonucleoprotein assemblies, including ribosome particles [73,85]. This helicase might overaccumulate in *clpr2-1* young and mature plants as a compensatory response to overcome a bottleneck in ribosome assembly or other aspects of plastid gene expression, or alternatively, because it is a substrate of the Clp protease system. Ongoing experiments favor the first explanation (unpublished data). RIF10/PNPase has been shown to be indispensable for 3′-end maturation of 23S rRNA transcripts and the efficiency of 3′ end processing of mRNAs and polyadenylation, as well as the degradation of mRNA and tRNA [130–132]. Consistent with its function in RNA processing, we observed PNPase in ribonucleoprotein-rich stromal fractions of around 1-3 Megadalton [73]. Processing of 23S rRNA depends on ribosome assembly and the increase in PNPase suggests that the delayed rRNA processing is not due to lack of PNPase, but reflects a compensatory reaction to other rate limiting steps, such as ribosome protein assembly. Interestingly, accumulation levels of chloroplast ribosomal subunits were not significantly affected, and indeed chloroplast protein synthesis was not blocked in the *clpr2-1* and *clpr4-1* mutants, as evidenced by detection of most of the chloroplast-encoded proteins [87,90]. Nevertheless, a strong and persistent upregulation of the BipA-type translation factor, EF-TU-1 and others in both mutants suggests specific effects on plastid translation, particularly on the elongation phase [87,90]. RH3 and several elongation factors (such as EF-Ts) also displayed increased accumulation in the antisense *clpP6* and in *clpr1-1* lines [81,91].

2.7. Identification of putative Clp protease substrates, substrate recognition, selection and delivery

Based on its relatively high abundance in undeveloped chloroplasts and more mature chloroplasts, and the central function of the Clp system in bacteria, it is expected that many chloroplast proteins can be substrates for the Clp protease system [12]. The challenge is to obtain direct evidence for chloroplast Clp substrates. *E. coli* and many other bacterial species have a unique trans-translation tagging system for recycling stalled ribosomes which involves an attachment of an ssrA tag of several amino acid residues to the C-terminus of the incompletely synthesized proteins for targeted degradation mainly by the Clp protease system (reviewed in [133,134]). In *E. coli*, ClpXP is responsible for more than 90% of the degradation of ssrA-tagged proteins and ClpAP and Lon degrading the rest (5% and 2%, respectively) [135]. In *B. subtilis*, ClpXP is also responsible for the turnover of ssrA-tagged proteins, but ClpCP does not seem to be involved at all [136]. Interestingly, in bacteria of the genus *Mycoplasma* which lack ClpP, ClpX and ClpA, the trans-translation tagging system is still preserved and ssrA-tagged proteins are processed by the Lon protease [137]. In photosynthetic organisms, ssrA sequences have been found in genomes of cyanobacteria [138,139] and in plastid genomes from the red lineage [140,141], but not in higher plant plastid genomes. Moreover, the reactivation of stalled ribosomes and the possible coupling to proteolysis has not been addressed in plant plastids. This is an important area that deserves attention, also given the importance of plastid translation in plastid-nuclear signaling (see further Section 2.9 below).

The bacterial Clp system is also involved in the degradation of N-end rule substrates. The N-end rule correlates the half-life of a protein to the nature of its N-terminal residues (reviewed in [142,143]). The N-end rule pathway in prokaryotes and eukaryotes exhibit similar features in the recognition of destabilizing N-terminal residues (designated as N-degrons) coupled with proteolysis but different key players are involved. In the cytosol and nucleus of eukaryotes, proteins harboring N-degrons are ubiquitylated and processed by the 26S proteasome [142,143]. In contrast, the prokaryotic N-end rule is executed through N-degron recognition by ClpS which subsequently delivers the substrates to the ClpAP complex for degradation (various aspects of this pathway are described in [53,58,144–146]). Recently, analysis of stability determinants of chloroplast-encoded proteins in tobacco suggests an N-end rule-like pathway in plastids [147]. This study involved systematic modification of the residue after the initiator methionine to test which of the 20 amino acids confer protein instability in the chloroplast. Results revealed that histidine and cysteine in the penultimate N-terminal position conferred the highest instability in plastids; in contrast, proline and threonine are the primary destabilizing residues in bacteria. It is not clear that these rules for plastid-encoded proteins also apply on the much larger set of nuclear-encoded chloroplasts proteins and it remains to be determined if a plastid N-end rule pathway employs the Clp system, or different proteases (e.g., Lon protease).

In our view, no solid and direct evidence for ClpPR substrates in the plastid has been obtained, even if several proteins have been suggested to be substrates. A dozen proteins were explicitly assigned as substrates based on increased accumulation as detected by image analysis of 2DE gels from chloroplasts of an antisense *CLPP6* mutant [81] or the *clpr1-1* mutant [91]. These proteins were mostly highly

abundant stromal proteins involved in e.g. protein synthesis or folding. Based on our analysis of *clpr2-1*, *clpr4-1* (described above) and a *clpp3* null mutant (unpublished), it is not clear that they indeed are upregulated because they are substrates; rather they seem to represent an indirect response to the Clp mutation. Examples are the increase in the plastoglobule protein Fibrillin 1A and the increase in the chaperones HSP90, HSP70 and CPN60 (see Sections 2.4 and 2.5 above).

As mentioned in the previous section, MEP pathway proteins have been suggested to be a direct substrate of the Clp machinery based on Western blot analysis of 5-day old seedlings of the *rif1* and *rif10* mutants impaired in plastid gene expression and/or RNA metabolism [148]. While this is certainly possible, an indirect link between MEP pathway protein levels and Clp activity seems quite likely, for three reasons: 1) the MEP pathway enzymes are under a strong developmental control (with highest expression in the youngest leaves) [149] and a comparison between very young mutant and wt seedlings without correction for the developmental delay can explain the relatively high levels of MEP pathway proteins in the mutants; 2) our *clpr2-1* studies provided an alternative scenario for upregulation of MEP pathway proteins under conditions where photosynthetic electron transport is impaired [87], and 3) our feeding experiments with IPP and DMAPP did not affect any of the *clppr* phenotypes, thus suggesting that loss of the MEP pathway (or the parallel cytosolic mevalonate pathway) is not the cause of these phenotypes. Moreover, the presence of chlorophyll a and b, and the elevated levels of carotenoids also show that none of these pathways are blocked.

Interestingly, it was shown that the ClpC1 chaperone is involved in the degradation of chlorophyllide a oxygenase (CAO) in *Arabidopsis* [150]. CAO is responsible for the conversion of chlorophyll a to chlorophyll b, in dependence of the amount of chlorophyll b [151]. Removal of the N-terminal domain (A domain) of CAO resulted in the overaccumulation of CAO and induced photodamage during greening [152]. In addition, it was found that the sequence 97-QDLLTIMILH-106 within the A domain of CAO comprised its N-terminal degron [153]. However, this N-degron did not confer protein instability when expressed in a chlorophyll b-deficient mutant (*chlorina 1-1*) indicating that chlorophyll b modulates CAO degradation [153]. How chlorophyll b increases the susceptibility of the A domain of CAO for degradation (by direct interaction or by mediating association through other protein factors) remains to be determined. Nevertheless, using the CAO N-degron sequence as a template, a similar degron in CP47 (9-GRLLAVHIMH-18) has been identified [153] suggesting that a general N-degron sequence might be recognized by a plastid-localized proteolytic system. We did not observe any significant changes in the chlorophyll a/b ratios for the mutants lacking ClpR2 and ClpR4, indicating that unregulated accumulation of CAO did not cause the phenotypes. In addition, a demonstration of the involvement of the ClpPR protease, rather than the ClpC1 chaperone with diverse functions, will be needed to further support CAO as a substrate of the Clp protease system.

The transcript analysis of different developmental stages and organs in *Arabidopsis* (Fig. 1) did not suggest any specific substrates, but it did suggest when Clp components were particularly important. For instance it suggested that ClpS and ClpT were expressed antagonistically during leaf development. A forthcoming large scale comparative proteomics study of maize leaf development showed that the Clp machinery has the highest abundance during the transition of proplastids to chloroplasts [79]. In contrast the abundance of other proteases, such as the thylakoid FtsH family, showed highest accumulation levels in mature chloroplasts, suggesting an important role in thylakoid maintenance and quality control. These general patterns of protease accumulation are important to suggest the most relevant context for more targeted substrate analysis.

Several alternative strategies for substrate identification can be envisioned, some of them have been successfully applied to the Clp system in *E. coli*. Trapping of substrates into a Clp complex, followed by mass spectrometry-based analysis of a purified Clp core with the trapped substrates, would be possible if the rate of proteolysis or release of proteolytic products can be sufficiently reduced. This has been a successful strategy for the *E. coli* ClpP–ClpX combination simply by mutation of one of the three catalytic residues (S97A) in the ClpP gene [154], but not yet for the ClpP–ClpA combination. However, because the plastid Clp core is composed of 9 different gene products (in one or more copies), most of which are essential, it remains to be seen if viable transgenic plants can be created that contain a ClpPR core with reduced proteolysis rates. An alternative strategy is to use the substrate selector ClpS as a starting point. Again, this strategy has allowed identification of more than 20 candidate substrates for the *E. coli* Clp system [155]. Similar experiments are underway for chloroplast ClpS and appear to provide numerous candidates as well (Asakura and van Wijk, unpublished results).

2.8. The Clp protease as part of a chloroplast protease network

The chloroplast contains multiple protease systems (Table 1) and it is likely that there are a number of proteins that can be degraded by more than one protease system. This has been demonstrated in bacteria for the Lon and Clp systems [135]. Moreover, degradation of substrates can also involve sequential proteolytic steps involving two or more protease systems operating in series. Examples are degradation of the thylakoid D1 protein involving both the FtsH and DegP system [16,24] and bacterial proteolysis in which oligopeptidase A degrades cleavage products generated by various proteases including the Clp complex [156].

Stromal Zn^{2+}-proteases PreP1,2 (also named ZnMP1,2) were suggested to be involved in degradation of cleaved chloroplast transit peptides [2,157,158] and were upregulated in the *clpr2-1* seedlings [87], but they were unchanged in the mature chloroplast in mature *clpr2-1* [86]. The increase of PreP could well reflect overlapping substrates with the Clp system, which would be most needed under conditions of chloroplast biogenesis and rapid protein import.

FtsH5 and FtsH2 (also named Var1 and Var2) and SppA were upregulated in mature *clpr2-1* plants [86,87]. This suggests an accelerated turnover of thylakoid proteins that might have accumulated in excess or are damaged. Several studies on suppression lines of variegation mutants deficient in an FtsH subunit (*var2*) have revealed a link between the FtsH and Clp protease systems (reviewed in [11]). Despite a uniform genetic background, the leaves of the *var2* mutant have both green or white/yellow sectors with wild type-like chloroplasts in the green sectors and with abnormal and vacuolated plastids in the white sectors [159–161]. Three *var2* variegation suppressor lines have been traced to mutations in Clp genes (Table 2). These include *clpC2* and *clpC1*, which lack respectively ClpC2 and ClpC1 [162], as well as *svr-2 (suppression of variegation2)* which is deficient in ClpR1 [127]. *svr2* and *clpC1* both suffer from defects in chloroplast rRNA processing [89,127] whereas *clpC2* does not [127]. The current model for variegation proposes a certain "threshold" of FtsH activity for normal chloroplast biogenesis during early leaf development which determines whether normal-appearing chloroplasts (green sectors) or mainly abnormal plastids accumulate (white sectors) [11]. With this model, several scenarios have been put forward to account for variegation suppression (reviewed in [11]). Although not observed in all *var2* suppressor lines, chloroplast rRNA processing defects might lead to reduced rate of chloroplast translation thereby delaying chloroplast biogenesis and providing more time for the threshold of factors to accumulate and compensate for *VAR2* deficiency. Reduced chloroplast translation might also alter transcription of nuclear genes encoding for plastid-targeted proteins through plastid-to-nucleus signaling (see Section 2.9). In another scenario, suppression arises from reduced translation of a chloroplast-encoded factor which inhibits chloroplast

biogenesis. The role of the Clp system in any of these scenarios remains to be characterized.

2.9. A role for the Clp protease system in plastid signaling to the nucleus?

The Clp core mutants have strong phenotypes, possibly stronger than expected from protease mutants; this could be due to pleiotropic effects or direct effects on plastid-nuclear signaling. Interestingly, the *CLPR2,4* null mutants were blocked in seedling development at the cotyledon stage, however this block could be removed by addition of sucrose to the medium [90]. Mutants with primary lesions in photosynthesis generally do not display this developmental block, suggesting that the Clp complex (or rather lack of the Clp complex) affects plastid-nuclear signaling. There is overwhelming evidence in the literature that a signaling pathway from plastid to nucleus exists, but the signal transduction pathway is poorly understood. Most crucially, no solid evidence for a mobile signal (traveling from plastid to nucleus) has been obtained. Candidate signals from intermediates in the tetrapyrrole pathway could ultimately not be confirmed—for discussions and references on this popular topic see [163–165]. However, there is a consensus that several events/processes within the plastid contribute to a signal; these include the ROS/redox state and plastid gene expression. The contribution of the lack of Clp protease to the signaling pathway could be via repression of plastid gene expression or via effects on metabolism, in particular due to isoprenoid derived molecules, such as abscisic acid (ABA) precursors down-stream of carotenoid synthesis. A more direct contribution of the Clp system to signaling could be that Clp substrate cleavage products export from the chloroplasts upon which signaling to the nuclear occurs through an uncharacterized pathway. While very speculative, such a scenario has been proposed to occur for the mitochondrial unfolded protein response in *Caenorhabditis elegans* based on some experimental evidence [166].

3. Conclusions and challenges

Intraplastid proteolysis is a key process for chloroplast development and maintenance. Whereas a few chloroplast proteases have been relatively well studied, the functional significance of many of the chloroplast proteins is not yet understood. The Clp protease system is the most abundant protease in the developing and mature chloroplasts. Genetic and proteomic studies of Clp mutants in Arabidopsis have established the importance of the Clp system in embryogenesis and plant development. The most urgent task ahead is to identify substrates and substrate recognition mechanisms for the Clp system and for all other chloroplast proteases; this will then allow determination of the complete plastid protease network.

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