Protein stability and degradation in plastids

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Abstract

Steady-state levels of chloroplast proteins rely on the balance between synthesis and degradation rates. Thus, the importance of protein-degradation processes in shaping the chloroplast proteome, and hence proper organellar functioning, cannot be overestimated. Chloroplast proteases and peptidases participate in chloroplast biogenesis through maturation or activation of pre-proteins, adaptation to changing environmental conditions through degradation of certain proteins, and maintenance of protein quality through degradation of unassembled or damaged proteins. These activities are mediated by ATP-dependent and -independent proteases, many of which are encoded by multigene families. Newly imported proteins are processed by stroma- and thylakoid-localized peptidases that remove signal sequences, which are then further degraded. The multisubunit ATP-dependent Clp and FtsH complexes degrade housekeeping and oxidatively damaged proteins in the stroma and thylakoid membranes, respectively. A number of other chloroplast proteases have been identified, but their function and substrates are still unknown, as are the nature of degradation signals and determinants of protein instability. Future research is expected to focus on these questions.

1 Introduction

The chloroplast proteome comprises more than 2000 nuclear- and chloroplast-encoded proteins. Steady-state levels of these proteins are determined by the balance between transcription and translation rates on the one hand, and degradation rates on the other. Thus, the importance of protein-degradation processes in shaping the chloroplast proteome, and hence proper functioning of the organelle, cannot be overestimated. Proteolytic activities, determined as cleavage of peptide bonds, are carried out by proteases or peptidases, which differ in a number of aspects. Some activities are limited to the hydrolysis of a single bond in a given substrate, whereas others function processively. Products of such activities can be either free amino acids or peptides of different lengths, from di- and tri-peptides to much longer ones. The hydrolysis itself can be catalyzed by different mechanisms, depending on the chemistry of the active site, giving rise to the categorization of proteases into seven different families based on the catalytic centers: serine, cysteine, aspartic, metalloproteases, threonine, glutamic, and peptidases of unknown catalytic mechanisms. Although cleavage of a peptide bond does not require

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metabolic energy, some proteases couple the hydrolysis of ATP to the unfolding of their substrates as a prerequisite for the actual cleavage of peptide bonds. The *in vivo* contexts of proteolytic reactions are also highly variable: maturation or activation of pre-proteins require either N- or C-terminal processing by specific peptidases; proteolytic enzymes participate in some cases of signal transduction by releasing factors from membranes into the soluble phase; rapid turnover rates of certain regulatory proteins allow their function as 'timing proteins' in the control of gene expression; protein quality control is maintained by the degradation of unassembled or damaged proteins. Thus, proteolytic processes are intimately involved in almost every aspect of the cell's life cycle. Organelles such as chloroplasts are no exception. Although examples have been documented for the involvement of only some of the above proteolytic processes in chloroplasts, it is already clear that proteases play an essential role in this organelle's biogenesis and function.

Looking back 25 years or so, research in the field of chloroplast proteolysis can be roughly divided into three periods. During the 1980s and early 1990s, a number of proteolytic processes were documented and characterized. However, attempts to identify the proteases involved in these processes, primarily through biochemical approaches, were largely unsuccessful. In the mid-1990s, the identities of the chloroplast proteases began to be revealed. These all turned out to be homologues of known bacterial proteases. Completion of the *Arabidopsis* genome project enabled comprehensive homology searches, and in conjunction with the use of programs for predicting the intracellular location of proteins, a list of putative components of the proteolytic machinery of chloroplasts was compiled (Sokolenko et al. 2002). Research in the field in recent years has been characterized by attempts to link identified proteases with the previously described proteolytic processes, and to reveal their physiological roles, primarily through a reverse-genetics approach.

This chapter reviews the different components of the chloroplast proteolytic machinery, the different proteolytic processes delineated to date in chloroplasts, and the limited information on determinants of protein stability and instability in chloroplasts. Where possible, proteolytic enzymes will be referred to according to their names and classification in the peptidase database MEROPS (Rawlings et al. 2006) (http://merops.sanger.ac.uk/index.htm) and its corresponding handbook (Barrett et al. 2004).

2 Major chloroplast proteases

Given the prokaryotic evolutionary origin of chloroplasts, it is not surprising that all chloroplast proteases are homologues of known bacterial ones. In fact, this relationship facilitated the initial identification of some chloroplast proteases. Proteases involved in intracellular proteolysis in any biological system can be categorized, based on their energy requirement, into ATP-dependent and -independent ones. Hydrolysis of a peptide bond does not require metabolic energy. Thus, the

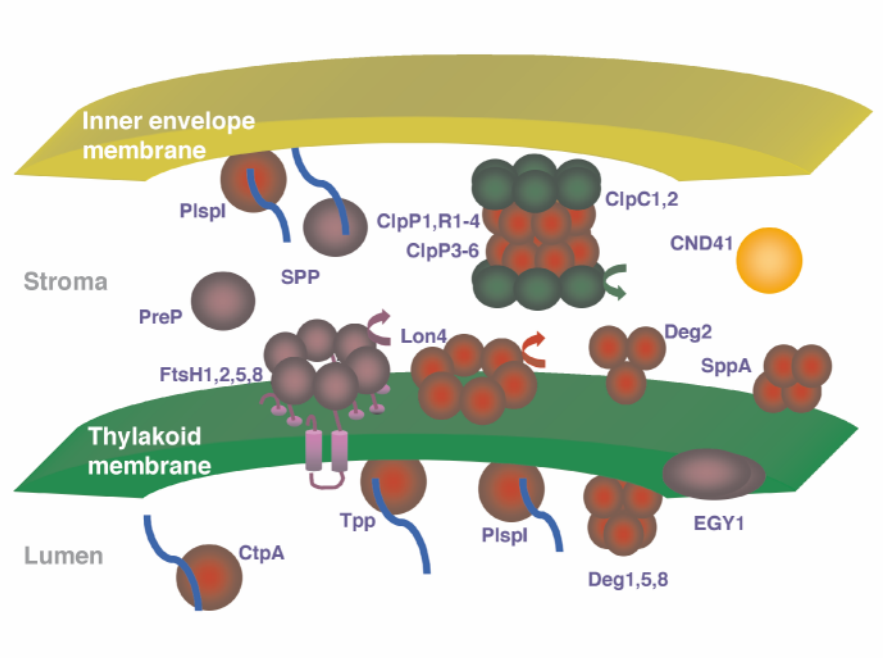

Fig. 1. Distribution and characteristics of chloroplast proteases. Serine proteases are depicted in red, metalloproteases are in purple, and the one aspartic protease is in orange. Arrows indicate ATPases that are found either together with the protease domain on the same polypeptide (FtsH and Lon proteases) or on a separate polypeptide (Clp protease). Ribbons attached to peptidases indicate precursor proteins processed by them.

requirement for ATP in certain enzymes is limited to unfolding the substrate and feeding it into a catalytic chamber, which is secluded from the cellular environment, a paradigm that led to classifying these enzymes as self-compartmentalizing proteases (Baumeister et al. 1998). Similar to all bacteria, chloroplasts contain both ATP-dependent and independent proteases. However, whereas *Escherichia coli* and most other bacteria contain single genes encoding these enzymes, higher plants have evolved multiple genes for most of them (Adam et al. 2001). These enzymes are described below.

2.1 Clp protease

Clp protease in *E. coli* is a multisubunit complex, composed of two main components, proteolytic, and regulatory (for review, see Sauer et al. 2004). The proteolytic chamber is made up of two heptameric rings of the serine peptidase ClpP. Together, they form a barrel-like structure with a narrow inlet and an internal cavity where the active-site subunits, composed of the catalytic triad of Ser-His-Asp, are located. The openings of the ClpP subcomplex are capped by hexameric rings

of specific ATP-dependent chaperones of the AAA^+ superfamily (Neuwald et al. 1999), either ClpA or ClpX, which recognize potential substrates, unfold them, and feed them into the catalytic chamber. ClpAP and ClpXP specifically degrade different regulatory proteins, and participate in protein quality control by degrading aggregated, misfolded and otherwise abnormal proteins (Sauer et al. 2004).

Chloroplast Clp protease is much more complex (for recent reviews, see Clarke et al. 2005; Adam et al. 2006). ClpP in *Arabidopsis* (peptidase S14.002) is encoded by six different genes, giving rise to proteins of 20 to 29 kDa, five of which are targeted to chloroplasts. Only one of these, ClpP2, is targeted to mitochondria where, together with ClpX, they form the mitochondrial Clp complex (Halperin et al. 2001b; Peltier et al. 2004). One of the ClpPs, ClpP1, is the only component of the chloroplast proteolytic machinery that is encoded in the organelle's genome. The *Arabidopsis* nuclear genome encodes four ClpP-like proteins, designated ClpR. These are similar in size and sequence to ClpP and located exclusively in chloroplasts, but they lack the conserved residues of the catalytic triad, and thus are not expected to perform a proteolytic function. The ClpP cognate chaperones in *Arabidopsis* include two copies of ClpC, the plant homologue of ClpA, and another related protein designated ClpD, all located in the chloroplast, and three ClpX proteins that are located in the mitochondria. Expression of all of these, with the exception of ClpD, appears to be constitutive under different short- and long-term stress conditions (Zheng et al. 2002). Additional Clp proteins include two copies of ClpS and one of ClpT. ClpS is unique to land plants, being absent from algae and cyanobacteria, and shares homology with the N terminus of ClpC (Peltier et al. 2001). ClpT is homologous to the *E. coli* ClpS (which shares no homology with the *Arabidopsis* ClpS), a substrate modulator of the bacterial ClpAP complex (Dougan et al. 2002), which is essential for the operation of the N-end rule pathway (see Section 6) in bacteria (Erbse et al. 2006).

Native isoelectric focusing followed by mass spectrometry revealed that the core of the chloroplast Clp protease is a complex of 325 to 350 kDa, composed of one to three copies of ClpP (ClpP1, ClpP3-ClpP6), four copies of ClpR (ClpR1-ClpR4), and one copy of ClpS (Peltier et al. 2001). Interestingly, the same core Clp complex is found in the stroma of chloroplasts and non-green plastids from roots and flowers (Peltier et al. 2004). More recent work, using native polyacrylamide gel electrophoresis followed by immunoblot analysis with specific antibodies for each of the Clp isomers, has shed more light on the structure of the core Clp complex. Two sub-core complexes were observed, probably corresponding to the two different rings. Whereas a 335-kDa core contained all chloroplastic ClpP and ClpR subunits, two smaller sub-complexes had different compositions: a 230-kDa complex contained ClpP1 and ClpR1-ClpR4, and a 180-kDa complex contained ClpP3-ClpP6 (Sjogren et al. 2006). How this asymmetrical distribution of subunits between the different rings affects the function of the Clp core remains to be determined.

Knockout and downregulation of Clp genes revealed some of the functions of Clp protease *in vivo*. Disruption of the chloroplast ClpP1 gene in tobacco resulted in loss of shoot development (Shikanai et al. 2001; Kuroda and Maliga 2003). Since there is still no reliable chloroplast transformation system for *Arabidopsis*, it

is not known whether inactivation of the ClpP1 gene in this species would be as detrimental as in tobacco. Inactivation or downregulation of several ClpP, ClpR and ClpC genes in *Arabidopsis* led to phenotypes of variable severity. Viable ClpP4 and ClpP6 knockout mutants could not be obtained, but repression of their expression by antisense constructs resulted in slow growth and a variegated 'yellow-heart' phenotype (Sjogren et al. 2006; Zheng et al. 2006). Yellow variegated leaves were also observed in rice as a result of disrupting the ClpP5 gene (Tsugane et al. 2006). Mutations leading to loss of ClpR1 (Koussevitzky et al. 2007) or a lower level of ClpR2 (Rudella et al. 2006) also resulted in a slow-growing, pale green phenotype. These results suggest that ClpR, although lacking a proteolytic site, is important for stabilizing the structure and/or regulating the function of the chloroplast Clp protease. However, details of ClpR's involvement are unknown.

Mutations in the regulatory ATPase have somewhat less severe consequences than mutations in ClpPs. ClpC1 mutants can grow autotrophically, but they are small and pale relative to wild type plants (Constan et al. 2004; Sjogren et al. 2004; Kovacheva et al. 2005). In contrast, a ClpC2 mutant is indistinguishable from the wild type (Park and Rodermel 2004), suggesting that the two copies of ClpC are redundant. It is not known why these two mutants have different phenotypes, but it might be due to different levels of accumulation of these two isomers, such that loss of the more abundant one has more severe effects.

Insights into the structure of the Clp core complex were also obtained from these mutants. The T-DNA line of ClpR2 was not a complete knockout. Instead, it contained lower levels of the ClpR2 transcript and protein (Rudella et al. 2006). Interestingly, this was accompanied by a decrease in the level of all other Clp core-complex subunits, demonstrating that they are all essential for the assembly and stability of the complex. Furthermore, the same analysis that suggested that the two heptameric rings have different compositions (Sjogren et al. 2006) was performed on the ClpP6 mutant; in the absence of ClpP6, the other components of the ring (ClpP3-ClpP5) did not accumulate, whereas components of the other ring (ClpP1 and ClpR1-ClpR4) did. However, only small amounts of the existing rings dimerized (Sjogren et al. 2006). Thus, it appears that each of the rings is stabilized only if it contains the full complement of its components, and that most, if not all of these components are not redundant.

2.2 FtsH protease

The *E. coli* FtsH is a membrane-bound ATP-dependent metalloprotease (for a recent review, see Ito and Akiyama 2005). Of all the ATP-dependent proteases in this organism, FtsH is the only essential one. Unlike Clp protease, its proteolytic and ATPase domains are found on the same polypeptide and not on separate ones. The N terminus of the protein contains two trans-membrane helices, which anchor the protein to the plasma membrane. This region is followed by the ATPase domain, which relates this protein to the AAA^+ superfamily (Neuwald et al. 1999). The proteolytic domain of the protein is found in the C terminus of the protein, and it contains the zinc-binding motif His-Glu-X-X-His, which serves as the cata-

lytic site of the protease. Similar to other ATP-dependent proteases, FtsH forms a hexameric ring-like structure, in which access to the proteolytic site is controlled by the ATPase domain. Details of these structural features were recently revealed when the three-dimensional structure of bacterial FtsH was determined (Bieniossek et al. 2006; Suno et al. 2006).

The FtsH gene family in *Arabidopsis* contains twelve members (for recent reviews, see Adam et al. 2005, 2006; Sakamoto 2006). Products of three of these (FtsH3, FtsH4, and FtsH10) are targeted to the mitochondria whereas the other nine (FtsH1, FtsH2, FtsH5-FtsH9, FtsH11, and FtsH12) are targeted to the chloroplasts, as revealed by transient-expression assays with GFP fusions (Sakamoto et al. 2003). Mass spectrometry analyses confirmed the presence of FtsH1, FtsH2, FtsH5 and FtsH8 in chloroplasts (Friso et al. 2004; Sinvany-Villalobo et al. 2004; Yu et al. 2004). Immunoblot analysis of isolated organelles suggested that whereas FtsH4 is located exclusively in the mitochondria, FtsH11 is dually targeted to both the mitochondria and chloroplasts (Urantowka et al. 2005). The chloroplast-targeted FtsH1 and FtsH5, FtsH2 and FtsH8, and FtsH7 and FtsH9 comprise three pairs of duplicated genes (see phylogenetic trees in Sakamoto et al. 2003; Yu et al. 2004; Adam et al. 2005). Of the four proteins that were indeed identified in chloroplasts, FtsH2 is the most abundant, followed by FtsH5, FtsH8 and FtsH1, in decreasing order of abundance (Sinvany-Villalobo et al. 2004). The differential abundance of these four FtsHs is positively correlated with the severity of phenotypes associated with mutations in the corresponding genes. FtsH2 mutants have variegated leaves, containing distinct green and yellow/white sectors (Chen et al. 2000; Takechi et al. 2000). Mutants in FtsH5 have only slightly variegated leaves (Sakamoto et al. 2002), whereas mutants in FtsH1 and FtsH8 are indistinguishable from wild type plants (Sakamoto et al. 2003). These mutant phenotypes suggest that FtsH might be involved in chloroplast biogenesis.

The size of the chloroplast FtsH monomer (peptidase M41.005) is ~74 kDa. It is located in the thylakoid membrane with its ATPase and proteolytic domains facing the stroma (Lindahl et al. 1996). It forms a complex of 400 to 450 kDa, which is probably a hexamer (Sakamoto et al. 2003; Yu et al. 2004). Several lines of evidence suggest that FtsH complexes are heteromeric: FtsH2 and FtsH5, identified by either specific antibodies or mass spectrometry, co-migrate on native gels, sucrose gradients and size-exclusion chromatography. Moreover, in mutants lacking one of these proteins, the level of the other is also reduced (Sakamoto et al. 2003; Yu et al. 2004). Although an authentic native FtsH complex has not yet been purified to homogeneity, insights into its composition can be obtained from overexpression experiments and analysis of single and double knockout mutants. Overexpression of FtsH8 compensates for the loss of its duplicated gene FtsH2 (Yu et al. 2004), and FtsH1 can compensate for the loss of its close homologue FtsH5 (Yu et al. 2005). However, attempts to restore the wild type phenotype by overexpressing FtsH5 in the FtsH2-mutant background were unsuccessful. Furthermore, double mutants of duplicated genes, either FtsH1 and FtsH5, or FtsH2 and FtsH8, were completely albino, and could grow only on agar plates supplemented with sucrose. In each of these double mutants, the presumably remaining FtsHs did not accumulate (Zaltsman et al. 2005b). Taken together, these results suggested that

the chloroplast FtsH complex is a hetero-oligomer composed of two types of subunits, each encoded by duplicated genes. Whereas subunits within a type are redundant, the presence of subunits from both types is essential for accumulation of the complex (Adam et al. 2005, 2006; Zaltsman et al. 2005b). Moreover, previous quantification of the different isomers (Sinvany-Villalobo et al. 2004) now suggests that the FtsH hexamer is composed of two subunits of 'type A'—FtsH1 and/or FtsH5, and four subunits of 'type B'—FtsH2 and/or FtsH8 (Zaltsman et al. 2005b; Adam et al. 2006). Why two types of subunits are needed for accumulation of the complex is not clear, but these conclusions, based primarily on genetic analyses, will have to be confirmed by a biochemical approach.

The FtsH protein does not accumulate in etiolated seedlings (Lindahl et al. 1996). Expression studies on the different FtsH genes demonstrated an increase in all of their transcript levels in response to short-term (2.5 h) exposure to high light intensity. Temperature shifts, to either high or low temperature, had almost no effect on FtsH transcript level (Sinvany-Villalobo et al. 2004). Interestingly, exposure to high light resulted in a transient decrease in the level of the FtsH protein itself (Zaltsman et al. 2005a). Thus, it is possible that the increase in FtsH transcript level in response to high light only compensates for the temporary loss of FtsH protein induced by this treatment, enabling its restoration to normal levels. Consistent with this view are recent findings from a proteomic analysis of the response to high light, where no increase in the level of chloroplast proteases or chaperones, including FtsH, was observed (Giacomelli et al. 2006). Differential spatial expression of different FtsHs can also be ruled out, as GUS-fusion experiments revealed similar patterns for FtsH1, FtsH2, FtsH5, and FtsH8 (Yu et al. 2004, 2005). However, FtsH transcript and protein levels appear to increase during the initial stages of *Arabidopsis* seedling development (Zaltsman et al. 2005a).

2.3 Lon protease

Another ATP-dependent protease in *E. coli* is Lon. Similar to FtsH protease, its ATPase and proteolytic domains are found on the same polypeptide. It is a hexameric serine protease that uses a Ser-Lys dyad in its active site (Botos et al. 2004), which is required for the degradation of abnormal as well as several short-lived regulatory proteins (for review, see Gottesman 1996). Plant homologues of Lon protease (peptidase S16.003) have been identified in mitochondria (Barakat et al. 1998; Sarria et al. 1998), but apparently they are also found in chloroplasts. Transient-expression assay of GFP fusions revealed that of the four genes found in *Arabidopsis*, products of Lon1 and Lon2 are targeted to the mitochondria and peroxisomes, respectively, whereas Lon4 is dually targeted to both the mitochondria and chloroplasts (Ostersetzer et al. 2007). Proteomic analysis of mitochondria revealed the presence of Lon3 in this organelle (Heazlewood et al. 2004; Heazlewood and Millar 2005). Moreover, immunoblot analysis of purified chloroplasts with an antibody against Lon1 revealed a cross-reacting protein of the correct size that was tightly associated with thylakoid membranes facing the stroma (Ostersetzer et al. 2007). This association is consistent with the previous localization of

plant Lon to the inner membrane of the mitochondria (Sarria et al. 1998), and the archaeal Lon to the plasma membrane (Fukui et al. 2002). Expression of Lon4 appears to be constitutive, as its transcript level did not change upon exposure to high light, or low or high temperatures (Sinvany-Villalobo et al. 2004). The oligomeric structure of the plant Lon protease, in chloroplasts or mitochondria, is not known.

2.4 Deg protease

The *E. coli* DegP (also known as HtrA) is an ATP-independent serine protease, peripherally attached to the periplasmic side of the plasma membrane, which is essential for survival at elevated temperatures (for review, see Clausen et al. 2002). DegP forms a hexameric complex, made up of two staggered trimers. Its monomer size is 48 kDa, composed of two distinct domains: the proteolytic domain, with a typical catalytic triad of Ser-Asp-His, is found at the N terminus. Two PDZ domains in tandem, implicated in protein-protein interactions, are located at the C terminus. In addition to its proteolytic activity, DegP demonstrates chaperone activity. Whereas the chaperone activity dominates at low temperatures below 22^{o}C, the proteolytic activity is manifested at elevated temperatures (Spiess et al. 1999). This transition between the two activities can be explained by the structure of the protein. At normal temperatures, the active site of the protease is blocked by segments of the protein itself. At elevated temperatures, a conformational change is induced, which makes the active site accessible to substrates (Krojer et al. 2002).

The *Arabidopsis* genome contains 16 genes homologous to DegP. These have been recently renamed Deg proteases (for a recent review, see Huesgen et al. 2005). Products of four of these have been identified in chloroplasts. Deg2 is found peripherally attached to the stromal side of the thylakoid membrane (Haussuhl et al. 2001), whereas Deg1, Deg5, and Deg8 are found in the lumen (Itzhaki et al. 1998; Peltier et al. 2002; Schubert et al. 2002). Size-exclusion chromatography demonstrated that recombinant Deg1 can form hexamers (Chassin et al. 2002). Nevertheless, the oligomeric structure of native Deg1 has yet to be determined. The presence of Deg1, Deg5, and Deg8 in the same compartment suggests their interaction, but this possibility still needs to be tested experimentally. Expression analysis has shown a fourfold increase in the transcript level of Deg1 and Deg8 upon exposure of *Arabidopsis* plants to a short-term high-light treatment, whereas temperature shifts had no effect (Sinvany-Villalobo et al. 2004). However, it is not known whether this increase is accompanied by a parallel increase in protein level.

2.5 Intramembrane proteases

Intramembrane proteolysis refers to a relatively recently discovered phenomenon, the cleavage of peptide bonds within trans-membranes helices. Such cleavage events are catalyzed by four groups of proteases: S2P, Presenilin, SPP, and

Rhomboid (for reviews, see Weihofen and Martoglio 2003; Wolfe and Kopan 2004). Two recent studies identified homologues of S2P in chloroplasts. A genetic screen for *Arabidopsis* mutants deficient in both chlorophyll accumulation and ethylene-induced gravitropism revealed EGY1, a 59-kDa membrane-bound metalloprotease that is located in the chloroplast (Chen et al. 2005). Although the intraorganellar location of EGY1 was not determined, mutant plants had reduced levels of granal stacks and light-harvesting complex (LHC) proteins, suggesting that this protease is required for proper chloroplast development. Another protease related to S2P, designated AraSP, was localized to the chloroplast inner-envelope membrane (Bolter et al. 2006). Antisense and T-DNA insertion lines of this protease demonstrated severely impaired chloroplast biogenesis. However, how these proteases are involved in chloroplast biogenesis is not clear. As for other intramembrane proteases, although homologous genes are found in plant genomes, and products of some of these are predicted to reside in chloroplasts, no reports on these have appeared yet.

3 Proteolytic processes in chloroplasts and the enzymes involved

3.1 Maturation of pre-proteins

Similar to all proteins synthesized in prokaryotic organisms, the 80 or so proteins synthesized within the chloroplast contain an N-formyl Met residue at their N terminus. Most of these proteins undergo maturation that involves two hydrolytic reactions: the N-formyl group is removed by peptide deformylase (PDF), and in most cases, this is followed by the activity of methionine aminopeptidase (MAP) (peptidase M24.001), which removes the N-terminal Met residue (Giglione and Meinnel 2001; Giglione et al. 2003). Thus, the activity of MAP has implications for the identity of the N-terminal residue of proteins encoded and synthesized in chloroplasts, and hence, might affect their stability through the N-end rule pathway (see Section 6).

Most chloroplast proteins are encoded in the nucleus and imported post-translationally into the organelle (see Chapter 11). A key feature in their targeting and import is their N-terminal transit peptide, which is cleaved off in the stroma during or shortly after import. This cleavage event is catalyzed by the stromal processing peptidase (SPP) (peptidase M16.004). SPP is a metalloprotease of ~140 kDa, containing the inverted zinc-binding motif HXXEH at its catalytic site (for a recent review, see Richter and Lamppa 2005). Recombinant SPP was shown to be able to cleave a wide range of chloroplast precursor proteins *in vitro* (Richter and Lamppa 1998), suggesting that SPP is the only enzyme responsible for this processing step. This contention was supported by *in vivo* studies in which expression of the single *Arabidopsis* SPP gene was downregulated by an antisense construct, resulting in lethal seedlings (Zhong et al. 2003).

After cleavage of the transit peptide, it remains bound to SPP. Its release from the enzyme is mediated by another cleavage event, carried out by SPP, which results in the release of two subfragments into the stroma. These subfragments are then further degraded by a soluble metalloprotease (Richter and Lamppa 1999). More recent work has suggested that degradation of the transit peptide is catalyzed by a 110-kDa metalloprotease designated pre-sequence protease (PreP) (peptidase M16.012; for a recent review, see Glaser et al. 2006). *Arabidopsis* contains two such genes, the products of which are dually targeted to both the chloroplasts and mitochondria, where they perform a similar function—degradation of the respective signal peptides (Bhushan et al. 2003). The crystal structure of this enzyme was recently determined, demonstrating that the active-site pocket is composed not only of the inverted zinc-binding motif HXXEH found in the N terminus, but also of more remote Arg and Tyr residues from the C terminus of the enzyme (Johnson et al. 2006). Identification and characterization of PreP as the protease-degrading signal peptide (Glaser et al. 2006) is consistent with the previous suggestion that a metalloprotease catalyzes this step (Richter and Lamppa 1999). However, since degradation of transit peptides by SPP and PreP has been studied mostly *in vitro*, it is not clear whether formation of two subfragments of the transit peptide by SPP is a prerequisite for further degradation by PreP. In any case, since PreP cleaves peptides in the range of 10 to 65 residues, but not shorter ones (Stahl et al. 2002, 2005), products of PreP activity must be further degraded by other as yet unidentified peptidases.

Nuclear-encoded proteins targeted to the thylakoid lumen are synthesized with a bipartite transit peptide. They are first processed by SPP in the stroma to yield an intermediate form, which is then translocated across the thylakoid membrane (see Chapter 11). This translocation step is followed by cleavage of the thylakoid-targeting sequence by the thylakoidal processing peptidase (TPP) (peptidase S26.008), a homologue of type I signal peptidase (for review, see Paetzel et al. 2002). TPP is an ~30-kDa membrane-bound serine protease that uses a Ser-Lys dyad for catalysis (Chaal et al. 1998). However, it is not known whether TPP further degrades the cleaved thylakoid-targeting sequence, or if complete degradation to free amino acids is catalyzed by other lumenal peptidases. Chloroplasts contain another homologue of this peptidase, designated plastidic SPase I (PlsP1) (Inoue et al. 2005). This peptidase was recently implicated in the maturation of Toc75, a component of the protein translocation machinery at the outer envelope membrane, which undergoes multiple maturation steps. Toc75 is first processed by SPP in the stroma, and then, after being targeted to the outer envelope membrane, cleaved again to yield the mature protein (Tranel and Keegstra 1996; Inoue and Keegstra 2003). This second step in the maturation of Toc75 is catalyzed by PlsP1 (Inoue et al. 2005). Interestingly, this peptidase has been localized to the envelope as well as to thylakoid membranes. Consistent with this dual localization was the observation that a PlsP1 knockout plant demonstrates accumulation of unprocessed forms of both Toc75 and the lumenal protein OE33 (Inoue et al. 2005).

Precursor processing is not limited to nuclear-encoded chloroplast proteins. The best characterized example of processing of a chloroplast-encoded protein is the D1 protein of photosystem II (PSII) reaction center. D1 is synthesized with a C-

terminal extension of unknown function. However, it is well established that this extension needs to be removed before the oxygen-evolving complex can assemble together with the core PSII complex (e.g. Taguchi et al. 1995). This maturation step is carried out by the C-terminal protease-2 (CtpA) (peptidase S41.002). CtpA is a 45-kDa soluble serine protease, which uses a catalytic Ser-Lys dyad located in the lumen (Inagaki et al. 1996; Yamamoto et al. 2001). To date, there is no evidence for its involvement in any proteolytic process beyond maturation of the D1 protein. Another chloroplast-encoded protein that is processed in the thylakoid lumen is cytochrome *f*. This protein is synthesized with an N-terminal extension. The function of this extension and the peptidase involved are unknown.

3.2 Adaptation to changing light intensities

Although light is essential to plants, it may also have detrimental effects on them, a phenomenon known as 'photoinhibition' (Barber and Andersson 1992). Plants have evolved a number of strategies to avoid the harmful effects of light on the photosynthetic machinery, and one of them involves proteolysis. Long-term adaptation to an increase in light intensity is accompanied by a decrease in the antenna size of PSII, leading to a decrease in the amount of excitation energy being funneled to the reaction center. This modulation of antenna size is achieved by proteolytic degradation of a subset of LHCII subunits (Lindahl et al. 1995; Yang et al. 1998). Several biochemical attempts to identify the protease involved in this process have been unsuccessful, but a recent report on a specific *Arabidopsis* mutant suggested the involvement of the FtsH6 protease (Zelisko et al. 2005). However, low sensitivity of the degradation assay and high variability within and between experiments suggested that further experimentation was needed before a firm conclusion could be made. Another proposed candidate for this activity is SppA, a homologue of the *E. coli* SppA protease (peptidase S49.001), which functions as a signal-peptide peptidase in bacteria. SppA is a thylakoid-membrane-bound, light-induced serine protease—characteristics which are consistent with its speculated role in LHCII degradation (Lensch et al. 2001). Interestingly, this is the only thylakoid protease whose level increased in response to high light (Giacomelli et al. 2006). Nevertheless, direct experimental support for the involvement of SppA in this process is still lacking.

The transition from high to low light is also accompanied by protein degradation. The best known example in this context is the 'early light-inducible protein' (ELIP). This protein, which is structurally related to LHCs, is rapidly degraded upon such a transition (Adamska et al. 1993). Despite its initial characterization (Adamska et al. 1996), the involved protease has not yet been identified.

3.3 Protein quality control

Accumulation of all major photosynthetic complexes requires coordination between the chloroplast and nuclear genomes. Although advances have been made in

recent years in understanding how these genomes communicate with each other (see Chapter by Bräutigam et al.), little is known about the mechanisms involved in regulating the correct stoichiometry between the different subunits of a given complex. In this context, it is assumed that fine-tuning of their levels is achieved by proteolytic degradation of super-stoichiometric subunits. First support for this assumption comes from a work published more than 20 years ago. Inhibition of protein synthesis in the chloroplast of *Chlamydomonas*, including that of the large subunit of Ribulose 1,5-bisphosphate carboxylase/oxygenase, resulted in degradation of the nuclear-encoded small subunit within the chloroplast (Schmidt and Mishkind 1983). These results suggested that unassembled subunits of a multiprotein complex are rapidly degraded. Indeed, similar observations have been made in other photosynthetic complexes as well. For instance, in *Chlamydomonas,* when cytochrome b_6, subunit IV and the Rieske protein of the cytochrome b_6-*f* complex cannot assemble with cytochrome *f,* they are rapidly degraded (Kuras and Wollman 1994). Similarly, a point mutation in the Rieske protein led to a significant decrease in its level, as well as the levels of other subunits of the cytochrome b_6-*f* complex. Crossing this mutant with one containing reduced levels of ClpP1 resulted in stabilization of these proteins, suggesting a role for Clp protease in the degradation of some unassembled proteins (Majeran et al. 2000).

In vitro studies have hinted at a role for Clp protease in the degradation of unassembled or abnormal proteins in the stroma as well. Mistargeting of the lumenal protein OE33 to the stroma resulted in its rapid degradation, with characteristics reminiscent of those of Clp protease (Halperin and Adam 1996; Halperin et al. 2001a). A similar function may be fulfilled by other proteases as well. Experiments with wild type or mutant forms of the Rieske protein have demonstrated that molecules that fail to translocate across the thylakoid membrane are rapidly degraded by a membrane-bound metalloprotease. This *in vitro* degradation reaction could be inhibited by increasing amounts of antibody against native FtsH (Ostersetzer and Adam 1997), suggesting that FtsH may be involved in protein quality control in the chloroplast as well.

Many mutants have been shown to contain lower levels of subunits in a complex when one other subunit is missing. This observation is often interpreted as degradation of the not-fully-assembled complex. However, such conclusions should be viewed with caution when no direct evidence for degradation is provided, for instance, through pulse-chase experiments. In some cases, translation of a complex's subunits is regulated by one component of the complex (a regulatory mechanism known as 'control by epistasy', see Minai et al. 2006 and references therein). Thus, lower levels of the subunits in a complex may result from reduced rates of translation in the chloroplast and not only from degradation of unassembled ones.

It has long been known that chloroplast proteins are unstable without their cofactors. For example, in the absence of chlorophyll, due to either inhibition of synthesis or mutation, chlorophyll-binding proteins are rapidly degraded (e.g. Kim et al. 1994 and references therein). Similarly, the lack of a single copper ion is sufficient to destabilize the electron carrier plastocyanin (Li and Merchant 1995). These observations suggest that minor structural changes, induced by a lack of

minor components of a protein, may render it susceptible to proteolysis. Nevertheless, although the above examples have long been known, the proteases involved in degrading these substrates remain a mystery.

3.4 Oxidatively damaged proteins

Not all light energy absorbed by the photosynthetic antenna is converted to chemical energy. Depending on environmental conditions, free radicals are generated in chloroplasts, and despite the presence of free-radical scavengers, chloroplast proteins are highly prone to oxidation processes, which may impair their structure and function. The best characterized oxidatively damaged protein in the chloroplast is the D1 protein of the PSII reaction center. Oxidative damage leads to its inactivation, and hence, to photoinhibition (for reviews, see Andersson and Aro 2001; Yamamoto 2001). A prerequisite for the repair of photoinhibited PSII is degradation of the D1 protein, and numerous attempts have been made to identify the protease(s) involved. Biochemical approaches have been largely unsuccessful. However, the identification of chloroplast proteases and availability of protease mutants have enabled testing the possible involvement of specific proteases in D1 degradation.

Attempts to test the potential involvement of FtsH in the degradation of D1 were first made using recombinant GST-FtsH1 (Lindahl et al. 2000). These experiments demonstrated weak albeit significant activity of the recombinant enzyme against the initial 23-kDa degradation product of the D1 protein, but not against the full-length protein. The weak activity and limited specificity may result from the homomeric nature of the recombinant enzyme used *in vitro*, as opposed to the heteromeric nature of the enzyme found *in vivo*, as described in Section 2.2. Variegated mutants of FtsH2 and FtsH5 provided an opportunity to test the possible involvement of FtsH protease in the repair cycle of PSII *in vivo* as well. These mutants demonstrated increased sensitivity to photoinhibition compared to the wild type, as revealed by PSII-activity measurements (Bailey et al. 2002; Sakamoto et al. 2002). Consistent with this proposed role was the finding that the D1 protein is stabilized, probably in an inactive form, in FtsH mutant plants after exposing them to photoinhibitory illumination (Bailey et al. 2002).

Deg2, associated with the stromal side of the thylakoid membrane, has also been implicated in D1 degradation. An *in vitro* study demonstrated that recombinant Deg2 could cleave the D1 protein at its stromal loop connecting the fourth and fifth trans-membrane helices, yielding an N-terminal 23-kDa product and a C-terminal 10-kDa product, suggesting that this protease participates in the initial stages of D1 degradation (Haussuhl et al. 2001). However, a recent *in vivo* study with mutants lacking Deg2 demonstrated a rate of D1 degradation under light stress that was comparable to the wild type, suggesting that Deg2 is not essential for D1 degradation (Huesgen et al. 2006).

A recent study suggested that Deg1, located on the luminal side of the thylakoid membrane, might also be involved in D1 degradation (Kapri-Pardes et al. 2007). Transgenic plants expressing a RNAi construct accumulated less Deg1,

were smaller than wild type and more sensitive to photoinhibition. These plants accumulated more of the D1 protein, probably in an inactive form, but less of 16- and 5.2-kD degradation products. Moreover, addition of recombinant Deg1 to inside-out thylakoid membranes could induce the formation of the 5.2-kD D1 C-terminal fragment *in vitro*. Taken together, these results suggest that Deg1 cooperate with proteases found on the stromal side of the membrane in the degradation of D1 protein during repair from photoinhibition (Kapri-Pardes et al. 2007).

A *Chlamydomonas* ATP synthase mutant has also been shown to lose PSII upon exposure to light. Crossing this mutant with a strain containing lower levels of ClpP resulted in stabilization of several PSII subunits, including the D2 protein, CP43 and CP47 (Majeran et al. 2001). It is not known whether this degradation process is identical to the one occurring in response to exposure to photoinhibitory conditions, or even whether the effect of Clp protease is direct or not. Nevertheless, these results suggest the involvement of soluble Clp protease in the degradation of membrane substrates as well.

4 Other functions

4.1 Nutrient stress and senescence

Nutrient stresses and senescence are both characterized by the need to remobilize internal cellular resources, some of which can be provided by the building blocks of existing proteins. Thus, massive protein degradation is expected to accompany the plants' attempts to deal with nutrient stress or their final developmental stage, senescence. However, only little is known about the involvement of specific proteases in these processes. Downregulation of ClpP1 in *Chlamydomonas* suggests involvement of the Clp complex in the degradation of thylakoid membrane proteins upon exposure to nutrient stress (Majeran et al. 2000). Nitrogen starvation results in degradation of subunits of the cytochrome b_6-f complex. However, in cells containing reduced levels of ClpP1, this degradation process is retarded, suggesting that Clp protease may be involved in the adaptation to nitrogen starvation via the degradation of existing abundant proteins.

Protein degradation in senescing leaves followed by nitrogen mobilization to younger ones is a well-documented phenomenon (Hortensteiner and Feller 2002). To date, one specific protease has been linked to the degradation of the most abundant protein in chloroplasts, Rubisco. CND41 (peptidase A01.050) is an aspartic protease of 41 kDa that is associated with chloroplast nucleoids and exhibits proteolytic activity against denatured Rubisco, among others (Murakami et al. 2000). Moreover, antisense plants demonstrated delayed senescence, along with stabilization of Rubisco as well as other chloroplast proteins (Kato et al. 2004). Interestingly, it was recently found that CND41 itself must undergo a protelytic processing step for its activation (Kato et al. 2005). The significance of CND41 binding to DNA is not yet known, but it could be a means of sequestering it from other chloroplast proteins. Degradation of plastid DNA during early stages of se-

nescence may release CND41 to the stroma, allowing the initiation of massive protein degradation.

4.2 Thermotolerance

A recent study on a thermosensitive *Arabidopsis* mutant suggests the involvement of FtsH11 in thermotolerance (Chen et al. 2006). This mutant was more sensitive to moderate high temperature than the wild type, whereas the FtsH2 and FtsH5 mutants were not. As a result, the FtsH11 mutant had lower photosynthetic capability than the wild type after exposure to 30^{o}C. Unlike the FtsH2 and FtsH5 mutants, the sensitivity of the FtsH11 mutant to high light was similar to that observed in wild type plants (Chen et al. 2006). These results suggest that the physiological functions of the FtsH2-FtsH5-FtsH8-FtsH1 complex and FtsH11 differ. In this context, it should be noted that FtsH11 is found in both chloroplasts and mitochondria (Urantowka et al. 2005), and it is impossible to conclude at this stage whether its contribution to thermotolerance is related to its activity in chloroplasts, mitochondria, or both.

5 Identification of specific substrates

The availability of specific protease mutants lends itself to the identification of their substrates, when these are unknown. Specific substrates of a protease are expected to be stabilized in a mutant background, and thus comparative proteomics has the potential to yield their unbiased identification. One successful utilization of this approach involved a comparison between stromal proteins in the wild type and ClpP6 mutant (Sjogren et al. 2006). Potential substrates of Clp protease found in this work included a nuclear exchange factor for the elongation factor Tu, the molecular chaperone HSP90, an RNA helicase, the folding catalyst PPIase, and the UPRT and NDP kinase proteins involved in nucleic acid synthesis. These results suggest that Clp substrates are more involved in chloroplast homeostasis than in metabolism (Sjogren et al. 2006). A similar approach should prove useful in the identification of substrates of other proteases as well.

6 Determinants of protein instability

Although progress has been made in identifying components of the chloroplast proteolytic machinery, and proteolytic processes have been documented, determinants of instability within the protein substrates themselves are still obscure. The N-end rule, discovered and characterized in eukaryotic cells, relates the half-life of a protein to the identity of its N-terminal residue. Proteins carrying a destabilizing residue at their N terminus are ubiquitinated and degraded by the 26S proteasome (Varshavsky 1992). The N-end rule was shown to operate in *E. coli* as well, where

degradation of substrates is mediated by the ClpAP protease (Tobias et al. 1991). The *E. coli* ClpS adaptor protein has been described as a modulator of ClpAP activity (Dougan et al. 2002), and more recently, shown to be essential for the operation of the N-end rule pathway in bacteria (Erbse et al. 2006). As plastids are descendants of a prokaryotic progenitor and many of their characteristics, including their proteolytic machinery, are prokaryote-like, it is highly likely that an N-end rule-like mechanism governs protein stability in plastids as well. As described in section 2.1, the plastid homologue of the bacterial ClpAP is designated ClpC. Plants also have a homologue of ClpS, known as ClpT (Peltier et al. 2004). However, ClpT was not identified in proteomic studies of the Clp core complex (Adam et al. 2006), and thus is not expected to be a component of the Clp protease core. Nevertheless, the presence of chloroplast homologues to components of the bacterial N-end rule pathway suggests that this pathway governs protein stability/instability in this organelle as well, a notion that awaits experimental validation.

The small stable RNA A (SsrA) system in *E. coli* tags proteins translated from incomplete mRNAs for degradation (Karzai et al. 2000). The ssrA RNA is a small molecule that acts as both tRNA and mRNA. When a ribosome stalls on an incomplete mRNA, the ssrA molecule binds the ribosome, which then reads through to add an 11-amino-acid long tag to the protein (a process referred to as 'trans-translation'). This tag contains the small nonpolar amino acid sequence Leu-Ala-Ala at its C terminus, which is recognized in the cytoplasm by the ClpAP, ClpXP or FtsH proteases, or in the periplasm by the DegP protease. Related sequences that can also be recognized by proteases are Leu-Val-Ala, Ala-Ser-Val and Ala-Ala-Val. To date, there is no evidence for the presence of ssrA RNA in plastids of higher plants (Gueneau de Novoa and Williams 2004). However, the presence of homologues of the bacterial proteases suggests that plastid proteins with C termini homologous to the SsrA tag would be short-lived. Moreover, even in the absence of an SsrA trans-translation system, the identity of C-terminal residues may confer stability or instability on a protein. However, as with the influence of the N terminus, this has not been explored to date.

7 Future prospects

Plant sequence data accumulated over the past 15 years, and completion of the *Arabidopsis* and rice genome sequencing projects, suggest that the identity of most, if not all chloroplast proteases and peptidases is now known. The major challenge ahead is to assign a function to each of them, and relate the proteases and peptidases to known proteolytic processes. A striking feature of many of the chloroplast proteases is the relatively large number of genes encoding them, compared with their prokaryotic progenitors. It is now clear that at least the Clp and FtsH proteases are heteromeric complexes, with little redundancy between their components. Assuming that ClpRs are indeed proteolytically inactive, it will be important to establish whether they have only a structural role, or perhaps other

functions, such as substrate binding or recognition. The apparent need for more than just one ClpR is also intriguing. Overexpression of different ClpRs in a specific ClpR mutant is expected to help resolve the question of their redundancy. A similar approach will be applicable to the study of the relations between the different ClpPs as well. Some of these questions are partially answered with respect to the FtsH protease. However, it is still important to understand why the FtsH complex requires two types of subunits for its accumulation. Relations between the three luminal Deg proteases also need to be sorted out. Do they form homo- or hetero-oligomeric complexes? Are they redundant or not?

Major insight into the functions of different chloroplast proteases has been gained using specific mutants. There are now a number of publicly available mutant collections, particularly T-DNA insertion lines, and well-established procedures, such as antisense and RNA interference, for downregulating the expression of specific genes or gene families. Thus, these will probably continue to serve as the main tools in deciphering the physiological functions of specific proteases. *In vitro* approaches will probably continue to complement these efforts. However, special attention should be paid to possible pleiotropic effects. Many mutants missing different chloroplast proteins demonstrate a similar phenotype: slow growth, reduced pigmentation, altered chloroplast morphology and reduced levels of thylakoids. Thus, efforts should be made to distinguish between these general effects and the specific function of a given protease leading to these effects. This requires more specific assays for specific proteolytic processes, better linkage to substrate proteins, and attempts to understand their involvement in a given physiological response.

Identification of specific substrates for each of the proteases will have to be accompanied by attempts to reveal recognition determinants. To date, an understanding of recognition mechanisms between chloroplast proteases and their substrates is almost totally lacking. This applies to both partners—the proteases and their substrates. Efforts will need to be made to identify subunits within a proteolytic complex, or domains within a given protease, that are responsible for substrate recognition and binding, and determinants on the substrates themselves that allow this recognition. All of these questions will keep the growing community of scientists interested in chloroplast proteases busy for years to come.

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