

# Cutting edge of chloroplast proteolysis

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**Chloroplasts have a dynamic protein environment and, although proteases are presumably major contributors, the identities of these crucial regulatory proteins have only recently been revealed. There are defined proteases within each of the major chloroplast compartments: the ATP-dependent Clp and FtsH proteases in the stroma and stroma-exposed thylakoid membranes, respectively, the ATP-independent DegP proteases within the thylakoid lumen and on both sides of thylakoid membranes, and the SppA protease on the stromal side of the thylakoid. All four types are homologous to proteases characterized in bacteria, but most have many isomers in higher plants. With such diversity, the challenge is to link the mode of action of each protease to the chloroplast enzymes and regulatory proteins that it targets.**

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Proteolysis is involved in a wide range of processes during the biogenesis and maintenance of chloroplasts. It should therefore be considered a vital homeostatic factor that influences metabolic functions such as photosynthesis under both optimal and adverse growth conditions. The degradation of specific proteins in different compartments of the organelle has been reviewed [1,2].

Many factors trigger the degradation of inherently stable chloroplast proteins, including changing light conditions, imbalances in the stoichiometry of multiple-subunit complexes, genetic mutations and limited availability of cofactors. Photoinhibition owing to increasing light intensities, for example, is accompanied by the degradation of several photosystem-II (PSII) proteins, especially the reaction-center D1 protein, as an integral component of the repair mechanism. Transitions from low to high light intensity also result in the degradation of chlorophyll-*a/b*-binding proteins to adjust the size of the photosynthetic antenna; the converse transition leads to the degradation of the early light-inducible protein (ELIP). For multimeric complexes, the absence of one protein subunit, owing to either mutation or perturbation of synthesis, leads to the degradation of the other subunits; this regulatory phenomenon is common to most major chloroplast protein complexes. Lack of prosthetic groups also leads to degradation of their cognate polypeptides [1,2].

In spite of these examples, extensive biochemical attempts to purify the chloroplastic proteases responsible have been only marginally successful. Only through alternative approaches, independent of the known substrates, have specific chloroplastic proteases recently been identified, and were all revealed to be homologs of defined bacterial proteases. The aim of this article is to describe our current view of the components of the chloroplast proteolytic machinery and their emerging roles in

chloroplast biology. For a recent review of processing peptidases and various aminopeptidases, see Ref. [3].

## Stromal Clp protease

Within the chloroplast stroma, the enzyme that is thought to be responsible for most protein degradation is the ATP-dependent Clp protease, a two-component protease that has been extensively studied in *E. coli*. Although direct proof for an active chloroplastic Clp protease remains slight, increasing correlative and circumstantial evidence strongly supports it. The complexity of chloroplast Clp proteins in higher plants is better understood by first describing the model Clp protease from *E. coli* [4,5]. This enzyme consists of an endopeptidase, ClpP (21 kDa), that relies on the unfolding activity of a molecular chaperone, either ClpX (46 kDa) or ClpA (83 kDa), to instigate protein degradation. This restriction on ClpP activity is sterically conferred. Two heptameric rings of ClpP form a barrel-like structure, with a single cavity (50 Å diameter) enclosing the Ser–His–Asp residues that constitute the catalytic triad of serine-type proteases [6]. The axial entrance apertures are considerably narrower (11 Å), preventing the entry of most folded polypeptides and thereby avoiding their inadvertent degradation [6]. Proteolysis requires the association of a single hexameric ring of the chaperone regulatory Clp subunit to one or both ends of the two-tier ClpP annuli [7]. Once bound, the chaperone partner confers substrate specificity, which differs between ClpX and ClpA, and delivers the unfolded protein via ATP hydrolysis into the proteolytic chamber of ClpP [8]. Once inside, the unfolded protein is quickly and indiscriminately degraded to small peptide fragments, which afterwards diffuse out. ClpA and ClpX also function alone as chaperones in the dissociation of specific multimeric proteins, and are now recognized as members of the Clp/Hsp100 chaperone family [9].

Clp proteins in plant chloroplasts were first recognized in 1990, with the discovery of a plastid-encoded gene corresponding to *E. coli clpP* [10] (Table 1). In most higher plants, this *clpP* gene (*clpP1*) is transcribed with two ribosomal protein genes (from the 5' end, *rps12* and *rpl20*) [11], although it is monocistronic in *Arabidopsis* and separated into three exons. There are five other distinct isomers of ClpP in *Arabidopsis*, which are all nuclear encoded and designated ClpP2–ClpP6 [12]. All ClpP isomers are localized within chloroplasts [13,14], except ClpP2, which is localized to mitochondria [15], although it might also be localized to chloroplasts [16].

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**Table 1. Summary of the major protease families in chloroplasts of *Arabidopsis***

Protein name	Size range (kDa)	Location <sup>a</sup>	Protein function	Protease type	ATP dependent
ClpP1, ClpP3–ClpP6	20–29	S, T	Proteolytic subunit	Serine	No
ClpR1–ClpR4	21–35	S, T	Unknown	Unknown	No
ClpC1, ClpC2	92	S, E	Regulatory subunit	–	Yes
ClpD	95	S	Unknown	–	Yes
FtsH1, FtsH2, FtsH5–FtsH9, FtsH12	66–81	Ts	Protease	Metallo	Yes
DegP1, DegP5, DegP8	25–35	TL	Protease	Serine	No
DegP2	60	Ts	Protease	Serine	No
SppA	68	Ts	Protease	Serine	No
Lon2, Lon3	91–98	?	Protease	Serine	Yes

<sup>a</sup>Abbreviations: E, envelope; S, stroma; T, thylakoid; L and s, lumenal and stromal side of the thylakoid membrane, respectively; –, no proteolytic activity.

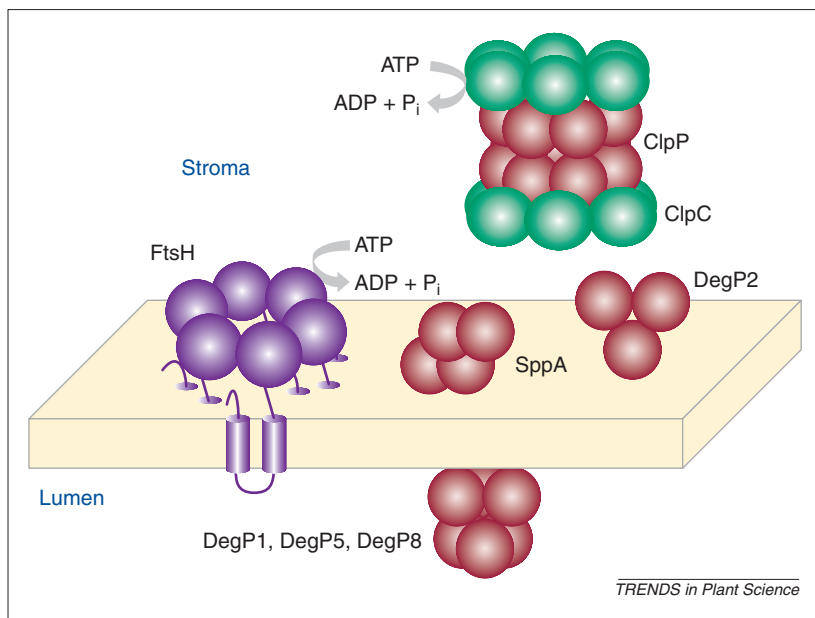
In addition to the ClpP isomers, there are two distinct regulatory ATPases inside chloroplasts: ClpC (92 kDa) and ClpD (95 kDa). Both are nuclear encoded and presumably have the unfolding activity needed to activate proteolysis by associated chloroplastic ClpP isomers. ClpC and ClpD have the two ATP-binding domains characteristic of group-1 Clp/Hsp100 proteins, which are essential for hexameric self-assembly and protein-unfolding activity. In higher plants, two separate nuclear genes produce ClpC, but they encode almost identical proteins and therefore are probably functionally indistinguishable. Previously, there was also thought

to be a ClpX ATPase inside chloroplasts, but it has since been found in mitochondria [15]. In association with ClpP2, this ClpX protein presumably forms a mitochondrial Clp protease in plants similar to that in mammals.

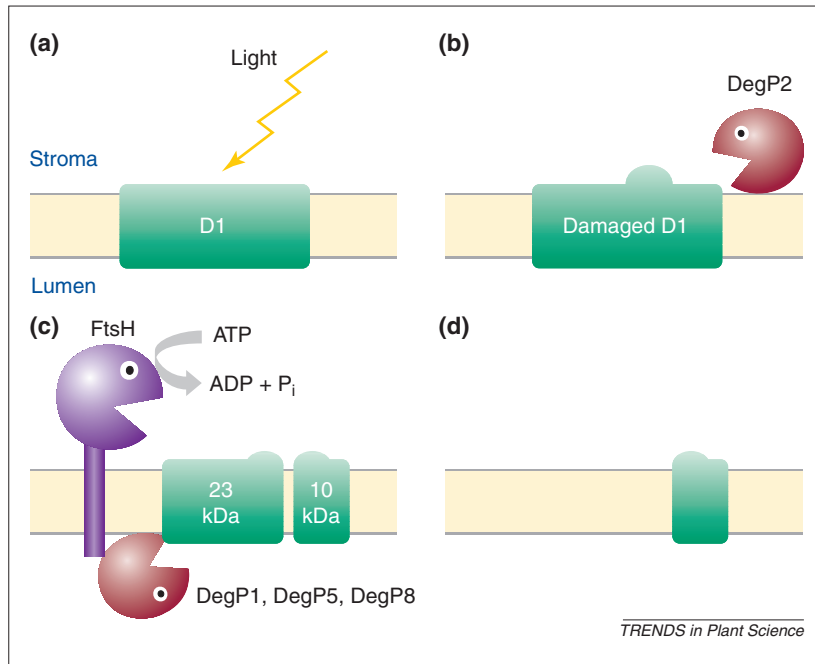
All chloroplastic Clp proteins are located in the stroma [14] (Fig. 1), although a small proportion of ClpP and of ClpC might also associate with stroma-exposed thylakoid membranes [16] and the inner envelope membrane [17], respectively. Most Clp proteins are synthesized constitutively in various plant tissues but are most abundant in chloroplasts from green leaves [14,18,19]. By contrast, the constitutive level of ClpD is relatively low but its gene expression is strongly induced by stresses such as dehydration, high salt, dark-induced etiolation, senescence and cold [14,20]. To date, prolonged low temperature is the only stress for which increased transcript levels translate into increased ClpD protein content in *Arabidopsis* leaves [14,21].

There is now evidence for structural associations between ClpC and certain ClpP isomers [13,22], suggesting that they form active stromal Clp proteases. The potential for this was shown *in vitro*, with proteolysis by recombinant plant ClpP1 being activated by *E. coli* ClpA and, conversely, plant ClpC activating the proteolytic activity of *E. coli* ClpP [18]. Recently, a functional stromal ClpCP protease has been isolated [23], although the identity of the associated ClpP isomers remains unclear. In the case of ClpD, there is currently neither structural nor functional evidence for its complexing to any ClpP isomer, and so its role in chloroplasts remains unresolved.

Although the exact role of stromal Clp proteases is unknown, they are evidently essential for chloroplast function because genetic interference with certain Clp proteins is detrimental to plant growth and viability. Inactivation of the *clpP1* gene prevents growth in algae [24]. The *clpP1* gene is essential for chloroplast development in tobacco [25], and no viable lines have been obtained with significant antisense repression of *clpC* [18]. In spite of this lack of causative evidence, a general function can be envisaged for chloroplast Clp proteins as housekeeping proteases. In this role, Clp proteases would facilitate the normal turnover of many stromal enzymes and regulatory proteins, as well as removing and recycling irreversibly damaged polypeptides. Such quality control is exemplified by the observation that incorrectly targeted proteins within the chloroplast are degraded in the stroma by enzymes with the characteristics of Clp proteases [26]. Other target proteins might be stromal enzymes such as Rubisco, whose subunits are adjusted proteolytically to maintain correct stoichiometry. Proteins within thylakoid membranes might also be subject to Clp proteolysis under certain conditions, as shown in algae for cytochrome *b<sub>6</sub>f* complexes during nitrogen starvation, mutated Rieske protein and PSII under light stress [27,28].



**Fig. 1.** Distribution of chloroplast proteases. The thylakoid membrane is shown in cream. The serine proteases ClpP, DegP and SppA are shown in dark red, and the metalloprotease FtsH in purple. The ATPases ClpC (green) and FtsH are indicated. The transmembrane helices of FtsH are shown as purple cylinders. The oligomeric forms of DegP proteins are suggested by the structure of the bacterial [49] and mitochondrial [50] homologs. It is currently not known whether ClpP, FtsH and DegP proteins form homo- or hetero-oligomers.



**Fig. 2.** A model for the degradation of the D1 protein of photosystem-II (PSII) reaction center.

(a) The D1 protein is damaged by light. (b) The damaged D1 protein undergoes a conformational change that makes it accessible to DegP2 residing on the stromal surface of the membrane. (c) Cleavage of the D1 protein by DegP2 yields 23-kDa and 10-kDa fragments. (d) The 23-kDa fragment has been degraded by FtsH. DegP1, DegP5 and DegP8 might facilitate degradation by cleaving portions of the protein exposed to the lumen.

New classes of Clp protein have recently come to light in higher plants, and constitute an intriguing variation on Clp proteolysis in other organisms. The first of these was originally identified in cyanobacteria and has been designated ClpR [29]. ClpR proteins are similar in size to ClpP and share low but significant primary sequence homology, but they lack the conserved amino acids that compose the serine-type proteolytic active site of ClpP [4]. There are four ClpR isomers in *Arabidopsis* [12], most of which are localized to chloroplasts [16]. Although their function remains unknown, certain ClpR isomers apparently associate with one or more of the stromal ClpP proteins [16]. Up to six other Clp-like proteins have also been identified in *Arabidopsis* [16], but little is yet known about their structural and functional characteristics.

#### Membrane-bound FtsH protease

The only essential ATP-dependent protease in *E. coli* is the membrane-bound FtsH [30] (Table 1). This metalloprotease facilitates the degradation of unassembled proteins and different regulators of gene expression [31]. Although there is only a single FtsH protein in most bacteria, yeast mitochondria have three distinct homologs – YTA10, YTA11 and YTA12 [32]. All three are attached to the inner mitochondrial membrane, where the YTA10–YTA12 complex faces the matrix and YTA11 the intermembrane space. One or two transmembrane hydrophobic domains located in their N-terminus anchor all FtsH proteases. Following this region is a conserved stretch of ~200 amino acids containing the Walker-A, Walker-B and SRH (second region of homology) motifs of the nucleotide-binding site, which includes this protein in the large family of AAA proteins [33]. The proteolytic site, the Zn<sup>2+</sup>-binding motif HExxH (where x represents any

amino acid), is situated towards the C-terminus of the protein.

Chloroplastic FtsH was first identified immunologically from spinach leaves, and characterized as an integral thylakoid membrane protein whose expression is light dependent [34]. The ~70 kDa protein is bound to the stroma-exposed lamellae, with its ATP- and Zn<sup>2+</sup>-binding domains exposed to the soluble stroma (Fig. 1). The predicted polypeptide in *Arabidopsis*, termed FtsH1, is similar to other FtsH proteins, especially in and around the functional domains [34]. A second FtsH isomer, FtsH2, was identified when a gene responsible for leaf variegation in the *Arabidopsis var2* mutant was isolated [35,36]. Overall, there are 12 different genes encoding FtsH proteases in the *Arabidopsis* genome, eight of which are targeted to chloroplasts, with the rest presumably being mitochondrial [3,12]. Four other highly homologous genes have also been found in *Arabidopsis*, but their Zn<sup>2+</sup>-binding domains are incomplete [3]. Thus, although they are proteolytically incompetent, these additional isomers might still fulfill chaperone functions because they retain the AAA domain.

The first indication of a functional thylakoid-bound FtsH came from an *in vitro* study. After import into isolated chloroplasts, unassembled Rieske Fe–S protein (which accumulated on the stromal face of the thylakoid membrane) and a soluble mutant of this protein (which accumulated in the stroma) were rapidly degraded [37]. Degradation was stimulated by light, probably owing to photodamage incurred by the unassembled protein. Characteristics of the degradation process were reminiscent of FtsH and, indeed, antibodies against the native protease could specifically inhibit degradation *in vitro* [37]. Another proteolytic activity attributed to thylakoid FtsH is the degradation of the PSII reaction-center D1 protein. It is well established that photosynthetic electron transport is inhibited under photoinhibitory conditions. Repair of the photodamaged PSII reaction center requires proteolytic removal of oxidized components (Fig. 2). The prevailing dogma is that the D1 protein is first cleaved into two fragments (10 kDa and 23 kDa) [2,38], presumably by DegP2 (see below) [39], with the 23 kDa fragment further degraded by a protease with the characteristics of FtsH [38] or recombinant FtsH1 [40]. Interestingly, these results suggest that, in some cases, initial cleavage of the substrate protein is a prerequisite for recognition and complete degradation by FtsH. Involvement of FtsH in D1 protein degradation was recently confirmed by analysis of the aforementioned FtsH2 (*var2*) mutant [41]. Although the fate of the 23 kDa fragment was not specifically examined, one can envisage a situation in which cleavage of the D1 protein is inhibited when further degradation of the 23 kDa fragment is prevented because of a mutation in FtsH. However, we cannot yet exclude the possibility of complete degradation of photodamaged D1 protein

by FtsH2. Whereas previously described substrates of FtsH in other organisms are either inherently short-lived regulatory proteins or unassembled proteins, the demonstration of FtsH involvement in D1 protein degradation defines a new class of FtsH substrates: functional proteins that have undergone irreversible oxidative damage.

The roles of FtsH protease appear to extend beyond protein quality control. In a study to identify plant genes involved in the hypersensitive response to tobacco mosaic virus infection, one of the transcripts whose level decreased before the appearance of necrotic lesions was *ftsH* [42]. Furthermore, in transgenic tobacco plants under- and overexpressing this gene, the size of necrotic lesions correlated with FtsH content; the lower the amount of FtsH, the more extensive the hypersensitive response [42]. It was speculated that reduced levels of FtsH inhibited efficient repair of PSII and thus inhibited photosynthesis, leading to cell death [42].

Ultrastructural analysis of the variegated *Arabidopsis* FtsH2 mutant leaves demonstrated underdeveloped plastids in the yellow and white sections, and normal chloroplasts in the green sections [35,36]. This suggests that FtsH protease is generally involved in chloroplast development, although its exact role in this process remains undefined. However, the patchy phenotype does imply that the loss of FtsH2 can be compensated for, at least partially, within the green, normal-looking leaf sectors. Because up to eight different FtsH proteins are localized to chloroplasts, they probably mediate any such compensation. Support for an FtsH role in biogenesis comes from cyanobacteria, in which loss of one of four *ftsH* genes reduced the level of functional photosystem I (PSI) by 60%, whereas PSII and phycobilisome content remained unchanged [43].

### DegP protease

*E. coli* has a family of serine-type ATP-independent proteases known as Deg, the first member of which, DegP (or HtrA), remains the best characterized [44] (Table 1). DegP is an enzyme extrinsically attached to the periplasmic side of the inner membrane [45]. It is a heat-shock protein that is essential for *E. coli* viability at high temperatures and is responsible for the degradation of misfolded or otherwise aberrant periplasmic and membrane proteins. Like the Clp and FtsH proteases, DegP incorporates chaperone and proteolytic activities; the chaperone activity dominates at low temperatures, whereas the proteolytic activity is present at elevated temperatures [46]. Other members of this family, DegQ (or HhoA) and DegS (or HhoB), are much less well defined but they all share the putative catalytic triad of serine-type proteases. An interesting structural feature of all these proteins is PDZ-like domains at their C-termini [44,47]. PDZ domains are implicated in protein-protein interactions in various

biological systems and apparently mediate substrate recognition and/or binding in the context of proteases [48].

The crystal structure of *E. coli* DegP was recently determined at high resolution [49]. It is a hexamer composed of two staggered trimeric rings. The proteolytic sites are located in a central cavity and the two PDZ domains of each monomer form the side walls. At low temperatures, the proteolytic site is inaccessible and thus probably inactive; only upon large conformational changes might it open [49]. This structure is therefore consistent with the lack of proteolytic activity of DegP at low temperatures and its increasing activity with increasing temperatures. The recent structure of a mammalian homolog in mitochondria differs from the *E. coli* protein in that it only has a single PDZ domain and forms a trimer. However, like bacterial DegP, its catalytic site is buried within the core of the enzyme [50].

In higher plants, a homologous protease (DegP1) is tightly bound to the lumenal side of the thylakoid membrane [51] (Fig. 1). It is produced constitutively but its level increases transiently in plants exposed to high temperatures [51]. The predicted *Arabidopsis* DegP1 has a putative bipartite transit peptide at its N-terminus, consistent with its localization to the lumen [51]. Although sequence conservation in the catalytic triad (His-Asp-Ser) and surrounding regions strongly support it as a plant DegP homolog, the predicted size of DegP1 and its single PDZ domain are more reminiscent of DegS. The exact role of a DegP1 protease is still unknown but serine-type proteolytic activity associated with the inner side of the thylakoid membrane has been demonstrated [51]. The location of DegP1 therefore makes it a strong candidate for a protease responsible for degradation of both soluble lumenal proteins and membrane-bound proteins with lumen-exposed regions. Interestingly, recombinant DegP1 (a mixture of monomers and hexamers) is proteolytically active towards both model and lumenal substrates, with a pH optimum of 6.5, and its activity increases with temperature [52].

Like other chloroplast proteases, DegP in *Arabidopsis* is encoded by multiple genes, of which at least four are putatively targeted to chloroplasts [12]. Experimental confirmation of this prediction was recently reported for the DegP1, DegP5 and DegP8 isomers, which were found in the lumen [53]. Whereas DegP1 and DegP8 are highly homologous, DegP5 shows high homology only in the catalytic domain and lacks an obvious PDZ domain. Given the high sequence similarity to the bacterial enzyme, it is expected that the chloroplast lumenal DegPs also form complexes, although it remains uncertain whether these will be homo- or hetero-oligomers.

A fourth DegP isomer, DegP2, was identified in chloroplasts as a peripheral protein attached to the stromal side of the thylakoid membrane [39] (Fig. 1). Increased production of this protein occurs under

high salt, desiccation and light stress conditions. Moreover, *in vitro*, this protease can cleave damaged D1 to 23-kDa and 10-kDa fragments [39] (Fig. 2), a prerequisite for complete degradation of this protein by FtsH (see above) before its replacement by a newly synthesized copy.

#### Other proteases

Another homolog of a bacterial protease, SppA, was recently identified in chloroplasts (Table 1). This is a serine-type, ATP-independent protease strongly bound to the stromal side of the thylakoid [54]. Plant SppA appears to be produced constitutively, but its level increases with increasing light intensity. This protease has been proposed to have a role in modulating the size of the photosynthetic antenna [54]. Chloroplasts apparently contain another ATP-dependent serine protease, a homolog of the bacterial and mitochondrial Lon protease. The *Arabidopsis* nuclear genome contains four Lon-encoding genes (Table 1). The products of two are located in mitochondria [55], whereas the others are predicted to target the chloroplast [12]. Recent studies have detected proteins cross-reacting to Lon-specific antibodies in chloroplasts (O. Ostersetzer and Z. Adam, unpublished), but little is yet known about the structure and function of these proteases.

#### Conclusions and future prospects

Specific proteases have now been identified in all major compartments of the chloroplast and, for most, genes coding for multiple isomers have been found. Such diversity among chloroplast proteases is shared by their cyanobacterial counterparts but differs from those in almost all non-photosynthetic prokaryotes, which commonly have only one form. The evolutionary forces that led to this diversity are not understood. Nevertheless, with the sequencing of the *Arabidopsis* genome, most (if not all) of the potential members of each chloroplast protease family are known. Although intracellular location can be predicted from sequence data, experimental evidence will show whether each of these putative proteases indeed resides within chloroplasts. Peptidases capable of limited proteolytic processing in the context of protein translocation, assembly or degradation also exist in chloroplasts [3], and their involvement in chloroplastic proteolysis will need to be addressed.

Most chloroplast proteases are expected to operate as oligomers, like their bacterial homologs. However, the presence of multiple isomers raises the question of what types of oligomeric structures exist in chloroplasts. Preliminary evidence, for example, suggests hetero-oligomeric complexes among some of the ClpP and ClpR isomers [16] in *Arabidopsis*. However, the exact conformations of these and other chloroplastic protease complexes have not been experimentally determined. The resolution of bacterial Clp protein structures [6,56] has also

revealed striking architectural similarities between the different ATP-dependent proteases. The eukaryotic 26S proteasome and the bacterial HslV/HslU and Clp proteases all share a cylindrical structure in which the proteolytic active sites are sequestered within the inner chamber and access to which is controlled by the cognate regulatory ATPases [57]. These chaperone subunits confer substrate recognition and binding, and then transfer the unfolded polypeptide into the proteolytic chamber. Other proteases such as FtsH also incorporate chaperone 'unfoldase' activity in addition to proteolytic activity, suggesting that this intricate regulatory mechanism is a conserved feature of crucial bacterial and eukaryotic proteases.

Although certain chloroplast proteases might degrade protein substrates indiscriminately, others are likely to be highly specific. Revealing such specific interactions between chloroplast proteases and their substrates remains a future challenge. The presence of C-terminal PDZ-like domains in many of the bacterial and chloroplast proteases suggests their possible involvement in substrate binding, but experimental data supporting this proposal are scarce. Other regions within prokaryotic proteases or their regulatory subunits might also contribute to or be solely responsible for substrate recognition and binding, and therefore identifying these should be a focus in the coming years. Understanding the mechanistic aspects of ATP-dependent degradation of integral membrane proteins also poses a special challenge. Although models suggesting how hydrophobic regions could be extracted from the membrane by the ATPase activity of the protease have been proposed [58,59], experimental confirmation is still lacking. This question is highly relevant to chloroplasts because thylakoid membranes are a major site of light-energy absorption and transduction, and the proteins within are highly prone to oxidative damage. In mitochondria, proteases on both sides of the inner membrane have been suggested to cooperate in the degradation of a monotopic model membrane protein [59]. The existence of proteases on both sides of the thylakoid membrane suggests that these might also cooperate in the degradation of multitopic membrane proteins, a possibility that should be investigated experimentally in the near future.

With the identification of specific chloroplast proteases, it is now possible to address specific substrates whose degradation has long been an enigma. Previous attempts to reveal the physiological roles of chloroplast proteases mainly used biochemistry. However, with current molecular techniques, modulating the levels of chloroplast proteases by transgenic techniques, identifying T-DNA insertion lines and analyzing the resultant mutants should further elucidate the links between known protein substrates and specific chloroplast proteases.

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