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REVIEW

# Chloroplast proteases

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## Abstract

The chloroplast within the plant cell has a dynamic environment where proteases play an important role in processing of precursor proteins, degradation of incomplete proteins lacking cofactors, stress-induced degradation and removal of damaged proteins. A number of proteases in the chloroplast are well characterized and found to be localized within different compartments such as stroma, thylakoids and lumen. In recent years, an increasing number of proteases in chloroplasts have been discovered and identified as bacterial protease homologues. These include the stromal Clp, thylakoidal FtsH and lumenal DegP. The current focus is to understand their role in chloroplast regulation both at the enzyme-substrate and genetic levels.

*Additional key words*: chloroplast lumen, protein degradation, proteolysis, regulation, stress, stroma, thylakoid.

## Introduction

Protein turnover occurs continuously throughout the life cycle of any cell. The steady-state content of all proteins is a result of balance between their synthesis and degradation. Proteases have been implicated in various plant processes such as germination, morphogenesis, senescence and programmed cell death (Huffaker 1990, Vierstra 1996, Beers *et al*. 2000). Proteolysis in plants has been reported in several cellular compartments such as chloroplasts, microsomes, mitochondria, and the Golgi apparatus (Palma *et al*. 2002). Nearly, 50 % of the total mass of mature chloroplasts is protein (Peoples and Dalling 1978). Much of the protein catabolism in chloroplasts takes place either in the early phase during its transition from proplastid to plastid or later during senescence. Proteolysis also helps in maintaining the functionality of the organelle both under normal and stress situations (Adam 1996). Proteolysis in chloroplasts in the past has been extensively reviewed (Adam 1996, Andersson and Aro 1997, Buetow 1997). In the past few years, researchers have tried characterizing a number of proteases by identifying bacterial homologues within the chloroplast (Lindahl *et al*. 1996). This article presents an overview of the current understanding of the proteolytic machinery present within the chloroplast and its role in regulation/maintenance of chloroplast stability.

Degradation takes place in different compartments of the chloroplast and generally in response to environmental stress, unavailability of cofactors and imbalance in the stoichiometry of multisubunit complexes. The substrates within the chloroplasts can be grouped into three different categories:

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*Abbreviations*: Chl - chlorophyll; CP43 - antenna-chlorophyll binding protein of photosystem 2; cyt - cytochrome; D1, D2 - reaction centre proteins of photosystem 2; LHC2 - light-harvesting complex 2; LSU - large subunit of Rubisco; OEC - oxygen-evolving complex; OEE - oxygen-evolving enhancer protein; PC - plastocyanin; PS1, PS2 - photosystems 1 and 2; RC - reaction centre; Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase; SSU - small subunit of Rubisco.

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**Substrates in stroma:** Non-stoichiometric amounts of multisubunit complexes result in rapid proteolysis. It was first shown in *Chlamydomonas* that when chloroplast protein synthesis (including that of large subunit of Rubisco, LSU) was inhibited, the newly imported small subunit (SSU) is rapidly degraded (Schmidt and Mishkind 1983). This degradation also occurs in cells deficient in chloroplast ribosomes suggesting the action of a nuclear-encoded constitutive protease (Schmidt and Mishkind 1983). In this process, a point mutation in LSU that prevents its assembly to a holoenzyme results in the failure of LSU and SSU to accumulate (Avni *et al*. 1989). Proteins mistargeted to the wrong compartments also undergo degradation. When the oxygen-evolving enchancer 33 kDa protein (OEE33) was intentionally mistargeted to the stroma instead of the lumen it was rapidly degraded (Halperin and Adam 1996). Environmental stress may also lead to degradation of stromal proteins. Rubisco becomes unstable when stress conditions are applied *in vivo* or *in vitro* (Mehta *et al*. 1992, Roulin and Feller 1997). Similarly, high irradiance increases instability of enzymes like glutamine synthetase, phosphoribulose kinase and nitrite reductase (Mitsuhashi and Feller 1992, Reinbothe *et al*. 1995).

**Substrates in thylakoid:** Imbalance in the stoichiometry of membrane complexes leads to the degradation of other subunits. The subunits of photosystem 1 (PS1) complex failed to accumulate when *psaC* was inactivated in *Chlamydomonas* (Takahashi *et al*. 1991). When the *psbK* and *psbO* were disrupted, the photosystem 2 reaction center (PS2 RC) complex was destabilized (Takahashi *et al*. 1994). A *Lemna* mutant lacking the Rieske Fe-S protein failed to accumulate the other components of the cyt$b_6f$ complex (Bruce and Malkin 1991). Even the non-availability of prosthetic groups affects the stability of chloroplast proteins. It was observed that the chlorophyll (Chl) *a/b*-binding protein failed to accumulate when the supply of new Chl was limited (Apel and Kloppstech 1980, Bennett 1981, Hoober and Hughes 1992). Chloroplast proteins also become susceptible to proteolysis due to their mode of function. For example, at the primary charge separation of PS2 RC the D1 protein is subject to photodamage with subsequent proteolysis (Mattoo *et al*. 1984). Similar light-mediated turnover of D2 and CP43 of PS2 has been reported (Schuster e*t al*. 1988, Christopher and Mullet 1994, Jansen *et al*. 1996). Photoadaptation in response to transition from low to high irradiance involves adjustments in the size of the photosynthetic antenna by specific degradation of LHC2 subunits to reduce the functional antenna size (Lindahl *et al*. 1995, Tziveleka and Argyroudi-Akoyunoglou 1998, Yang *et al*. 1998). Degradation of early light inducible protein (ELIP) that is structurally related to LHC2 was observed on transition from high to low irradiance (Adamska *et al*. 1996).

**Substrates in lumen:** Degradation of thylakoid membrane proteins exposed to the lumenal side has been demonstrated. The truncated forms of oxygen-evolving complex 23 kDa protein (OEC23) are correctly targeted to the lumen where they are processed to their mature size but are rapidly degraded (Roffey and Theg 1996). Earlier, it was shown that when *Chlamydomonas* cells were grown in a $Cu^{2+}$-deficient medium, apoplastocyanin (apo-PC) fails to accumulate due to rapid degradation (Merchant and Bogorad 1986). Even *in vitro* apo-PC is sensitive to proteolysis, unlike the mature or reconstituted holoprotein, which is insensitive (Li and Merchant 1995).

In addition, to proteolysis of specific substrates in chloroplastic compartments, post-translational cleavage of precursor proteins takes place during or after import into the chloroplast from the cytosol (Cline and Henry 1996). The thylakoid processing peptidase cleaves proteins destined for the thylakoid lumen after their translocation (Robinson *et al*. 1998). Even plastid-encoded proteins such as D1 proteins of PS2 RC and cyt*f* of cyt$b_6f$ complex undergo proteolysis to remove their carboxy or amino terminal extensions. These extensions are further degraded to free amino acids for reutilization (Van't Hof and de Kruijff 1995).

## Chloroplastic proteases in stroma

**CPE:** Import of proteins into the stroma is complete only after removal of the transit peptide. This reaction is catalyzed by the chloroplast processing enzyme (CPE) which is a metalloprotease containing the Zn-binding motif (Oblong and Lamppa 1992, Vandervere *et al*. 1995). This enzyme functions as a general processing peptidase and removes stromal targeting peptides from all imported proteins (Richter and Lamppa 1998). The loss of the CPE cannot be compensated by other stromal enzymes and chloroplast biogenesis is disrupted *in vivo* when an antisense to CPE is constructed (Wan *et al*. 1998). Another processing enzyme that can cleave a number of chloroplast precursors has also been found to be a 80 kDa metalloprotease (Koussevitzky *et al*. 1998).

**Clp:** Clp protease was discovered in *Escherichia coli* and is composed of two functionally distinct subunits: ClpP and ClpA or ClpX (Wickner *et al*. 1994, Porankiewicz *et al*. 1999). Clp proteins in plant chloroplasts were first identified in 1990 and represents a unique family of serine proteases encoded in the plastid (Gray *et al*. 1990, Maurizi *et al*. 1990), expressed constitutively (Shanklin *et al*. 1995, Ostersetzer and Adam 1996) and located within the stroma (Zheng *et al*. 2002). There are five

distinct nuclear-encoded isomers of ClpP in *Arabidopsis* (Adam 2001). The chloroplast also posses two different homologues of ClpA, designated ClpC and ClpD. ClpC appears to be the main Clp/Hsp 100 protein in plant and is constitutively expressed (Shanklin *et al*. 1995, Ostersetzer and Adam 1996). ClpC was first identified in tomato (Gottesman *et al*. 1990) and later in pea (Moore and Keegstra 1993). The ClpC can function on its own and has been found to be associated with chloroplast inner membrane import machinery (Nielsen *et al*. 1997). The ClpD protein is also synthesized constitutively but to a lesser extent than ClpC (Zheng *et al*. 2002) However, it has been shown to be strongly induced by dehydration, high salt concentration, dark-induced etiolation, senescence and cold conditions (Nakashima *et al*. 1997, Zheng *et al*. 2002). Although exact role of Clp proteases remains unknown, it can be thought of a housekeeping protease that facilitates removal and recycling of damaged proteins or targets stromal enzymes and regulatory proteins. For example, degradation of incorrectly processed precursors imported from cytosol by enzymes with characteristics of Clp protease within the stroma (Halperin and Adam 1996). Other potential targets may be Rubisco, whose subunits are proteolytically adjusted to maintain correct stoichiometry (Schmidt and Mishkind 1983). Clp proteolysis also facilitates protein degradation within thylakoid membranes under certain conditions, as shown in algae for cyt$b_6f$ complexes during $N_2$ starvation, mutated Rieske protein and PS 2 under light stress (Majeran *et al*. 2000, 2001).

## Proteases in thylakoids

**Thylakoid processing peptidase:** Chaal *et al*. (1998) characterized and cloned the thylakoid processing peptidase from *Arabidopsis thaliana* which cleaves leader sequences of proteins targeted to the lumen. The enzyme contains a Ser-Lys dyad and is similar to Type I leader peptidase from *Escherichia coli*, cyanobacteria and yeast mitochondria.

**FtsH:** It is the only ATP-dependent metalloprotease in *Escherichia coli* (Herman *et al*. 1993) involved in the degradation of transcription factor $\sigma^{32}$ (Tomoyasu *et al*. 1995) and unassembled SecY (Kihara *et al*. 1995). A homologue of this protein was detected in thylakoid membrane of spinach chloroplast (Lindahl *et al*. 1996). Chloroplastic FtsH is an integral thylakoid-membrane protein whose ATP- and $Zn^{+2}$-binding domains are exposed to the stroma. It was shown to degrade unassembled Rieske FeS protein that accumulates on the stromal face of thylakoid membrane after its import *in vitro* (Ostersetzer and Adam 1997). The FtsH is also involved in the degradation of PS2 reaction centre D1 protein following photoinhibition. Recovery from photoinhibition requires proteolytic removal of oxidized components. D1 protein is first degraded into two fragments (23 kDa and 10 kDa) (Spetea *et al*. 1999). The 23 kDa fragment is further degraded by FtsH in an ATP-dependent way (Lindahl *et al*. 2000). Analysis of *var2* mutant in *Arabidopsis thaliana* confirmed the degradation of 23 kDa protein by FtsH (Bailey *et al*. 2002).

**SppA:** SppA is a serine-type, ATP-independent protease identified in chloroplast as a bacterial homologue. It is strongly bound to the stromal side of the thylakoid. Its content increases with increasing irradiance and appears to have a role in modulating photosynthetic antenna size (Lensch *et al*. 2001).

## Enzymes in lumen

**DegP:** DegP belongs to a class of periplasmic serine-type ATP-independent proteases in *Escherichia coli*. It is a heat-shock protein; essential for survival at high temperature (Lipinska *et al*. 1990). DegP acts as a chaperone at low temperatures, whereas at elevated temperatures it acts as a protease. In higher plants, a homologous protease (DegP1) is tightly associated on the lumenal side of thylakoid (Itzhaki *et al*. 1998). It is constitutively expressed and its level increases transiently on exposure to higher temperatures. The exact role is unknown but serine-type activity was shown (Itzhaki *et al*. 1998). Recombinant DegP1 has been shown to be active at pH 6.5 against lumenal substrates (Chassin *et al*. 2002). Thus, the location and activity of DegP1 makes its involvement in lumenal loop degradation of D1 protein a strong possibility. DegP2 is also a peripheral protein associated with the stromal side of the thylakoid membranes (Haussuhl *et al*. 2001). Its expression increases under stress conditions and *in vitro* it can cleave damaged D1 to 23 kDa and 10 kDa fragments (Haussuhl *et al*. 2001).

**Tsp:** Tsp is a bacterial tail-specific periplasmic protease which is capable of processing certain substrates or even totally degrade other substrates (Silber *et al*. 1992). A chloroplast homologue of this protein, CtpA is found in the lumen (Inagaki *et al*. 1996, Oelmuller *et al*. 1996). It is involved in pre-D1 processing and removes the carboxy terminal of the pre-D1 protein.

## Conclusion

Studies on chloroplast proteases were initially prompted by investigation on the senescence of leaves (Buetow 1997). This got an impetus when it was reported that recovery from photoinhibition involves the proteolytic removal of the photodamaged D1 protein (Mattoo *et al*. 1984). The last decade has seen a tremendous progress in the demonstration of proteases within plastids (Adam 2000). The advancement in molecular biology has contributed a lot in the identification of bacterial homologues of chloroplast proteases (Lindahl 1996). Most known chloroplast proteases appear to be expressed constitutively and intensive search for certain environmentally induced proteases have been unsuccessful. But, recent studies from our laboratory show that there can be inductive mechanisms operating under certain extreme stress conditions especially in case of serine-type of activities (Nair and Ramaswamy, unpublished). Unlike thought earlier, even conventional methods of chloroplast and protein biochemistry are yielding results, as seen in the identification of proteolytic activity against LHC and D1/D2 core proteins of PS2 (Nair and Ramaswamy 2001, Georgakopoulos *et al*. 2002). However, the number of proteolytic pathways involved remains largely unknown. There still exists a large gap between characterizing an activity and identifying its physiological substrate. This broadens not only the involvement of multiple pathways that can exist/co-exist (action on a single/particular substrate) but also the nature of regulation that comes into play. There are still certain issues that need to be addressed such as the possible role of proteases in the regulation of gene expression at chloroplast (cellular) level. Similarly, confirmation of the location of putative proteases predicted from gene sequence data, study of configuration and mechanistic aspects of these protease complexes is required. The possibility of specific protease inhibitors *in vivo* regulating such activities should not be overlooked. The control of proteolytic machinery will also be of interest in terms of increasing plant productivity through agricultural biotechnology, especially under stress situations. Thus, the strategy of delaying/manipulating senescence in order to keep chloroplasts (leaves) photosynthetically active for longer periods will help boost crop yields. Little is known about programmed cell death (PCD) in plants. Considering senescence a type of PCD, the study of organelle proteases could provide deeper insights into this process. Coming years will focus on the above mentioned areas for better understanding the significance of chloroplast proteases at a molecular level and their application for crop improvement. Long thought to be an enigma and just about a few years back considered to be in its infancy, we believe chloroplast proteases now are slowly but finally coming out of age!

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