Chapter 20

Plastid Protein Degradation During Leaf Development and Senescence: Role of Proteases and Chaperones

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Summary

During leaf development, plastids undergo dynamic changes in morphology. Chloroplasts develop from proplastids during leaf growth: this process includes synthesis, import, and maturation of numerous chloroplast proteins. During leaf senescence, chloroplasts change gradually into a senescing form termed gerontoplast, with the breakdown of thylakoid membranes and the degradation of photosynthetic proteins. In these developmental processes, it is apparent that the proteolytic activity within chloroplasts is a key to control such remarkable morphological/functional changes. Processing and maturation of chloroplast proteins are very important since chloroplast development requires numerous proteins that are imported from the cytosol. Various efforts to elucidate the functions of chloroplast proteases have revealed the existence of signal peptidases (SPP, PreP, TPP, and PlsP1) that are involved in the processing and the maturation steps. In addition, the quality control of proteins is

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necessary for proper chloroplast development. Recent studies using Arabidopsis mutants have identified several important chloroplastic proteases (Clp, FtsH, Deg, and some intramembrane-proteases), which originated from bacterial homologs, in the quality control of proteins during chloroplast development. In contrast, studies on the degradation of chloroplast proteins during senescence implied that multiple pathways, not limited to chloroplast proteases, control protein degradation in this process. In addition to protein degradation inside the chloroplasts, degradation of engulfed whole-chloroplasts within the vacuole, and small spherical bodies like senescence-associated vacuoles (SAV), and Rubisco-containing bodies (RCB) that include chloroplast stromal proteins are known to occur during leaf senescence. The latter implicates that autophagy plays an important role in delivering chloroplast proteins into the vacuole. This chapter provides an integrated summary on the roles of chloroplast proteases during chloroplast development, and the current view of the chloroplast protein degradation during senescence.

I. Introduction

Along with protein synthesis, proteolysis is necessary for biological activities. Because, proteases are involved widely in various activities of living cells: they play important roles in proper protein maturation, quality control of proteins, and unnecessary protein degradation. In cellular organelles such as chloroplasts, proteases play crucial roles during change in the plants' morphology and function dynamically in response to leaf developmental status and environmental signals. During leaf development, rapid differentiation of proplastids into chloroplasts occurs; during leaf senescence, chloroplasts change gradually into a senescing form designated as gerontoplasts (Mulisch and Krupinska, Chap. 14; Lichtenthaler, Chap. 15). These conversions of chloroplasts accompany dynamic changes in the composition of chloroplast proteins.

To date, more than 16 kinds of proteases have been identified in chloroplasts using various experimental and in silico approaches (Kato and Sakamoto 2010); the proteases that have been identified in Arabidopsis chloroplast are listed in Table 20.1. Chloroplasts are evolved from a cyanobacterial ancestor by a primary endosymbiotic event 1 billion years ago (Archibald 2009). It seems reasonable to consider that the vast majority of these proteases are homologs of known bacterial proteases. Mounting evidence indicates that many of these proteases are actually involved in the processing and maturation of polypeptides, and in the quality control of proteins during plastid differentiation and chloroplast homeostasis. In contrast, the participation of these proteases in degrading chloroplast proteins during leaf senescence is poorly understood. During leaf senescence, in addition to protein degradation inside chloroplasts, protein degradation in other organelles is known to occur. Results of recent studies imply that multiple

Abbreviations: AAA – ATPases associated with diverse cellular activities; ATG – Autophagy-related gene; Cpn60 – Chaperonin 60; cpSec – Chloroplast secretory pathway; cpSRP – Chloroplast signal recognition particle; cpTat – Chloroplast twin-argnine translocation; GroEL – Hsp60 class oligomeric molecular chaperone; Hsc – Heat shock cognate protein; Hsp – Heat shock protein; LHC – Light-harvesting complex; MEP – Methylerythritol phosphate; OEC – Oxygen-evolving complex of photosystem II; PAO – Pheophorbide *a* oxygenase; Plsp – Plastidic type I signal peptidase; PPH – Pheophytin pheophorbide hydrolase; PreP – Presequence protease; PSII – Photosystem II; RCB – Rubisco-containing body; Rubisco – Ribulose 1,5-bisphosphate carboxylase-oxygenase; SAG – Senescence-associated gene; SAV – Senescence-associated vacuoles; SPaseI – Type I signal peptidase; SPP – Stromal processing peptidase; SREBP – Sterol-regulatory element binding protein; TIC – Translocon at the inner envelope membrane of chloroplasts; TOC – Translocon at the outer envelope membrane of chloroplasts; TPP – Thylakoidal processing peptidase

Table 20.1. Proteases in Arabidopsis plastid

Protease type	Clan	Family	Protein	Gene name	Location	Protein property	Mutant phenotype	References
Serine	PA	S1	**Deg protease**					
			Deg1	At3g27925	T (l)	PDZ domain	No knockouts reported (Lethal?)	Kapri-Pardes et al. 2007 Sun et al. 2010b
			Deg2	At2g47940	T (s)	PDZ domain	No phenotype	Huesgen et al. 2006
			Deg5	At4g18370	T (l)	–	High-light sensitive	Sun et al. 2007
			Deg7	At3g03380	T (s)	PDZ domain	High-light sensitive	Sun et al. 2010a
			Deg8	At5g39830	T (l)	PDZ domain	High-light sensitive	Sun et al. 2007
			Deg11[a]	At3g16540	–	PDZ domain	–	–
	SF	S26	TPP	At2g30440	T (l)	Membrane-bound	–	Chaal et al. 1998
	SF	S26	Plsp1	At3g24590	E/T	–	Seedling lethal	Shipman-Roston et al. 2010
	SJ	S16	Lon4	At3g05790	T (s)	AAA	–	Ostersetzer et al. 2007
	SK	S14	**Clp protease**[b]					
			ClpP1	AtCg00670	S/T (s)	Clp protease core	No knockouts reported (Lethal?)	–
			ClpP3	At1g66670	S/T (s)	Clp protease core	No knockouts reported (Lethal?)	–
			ClpP4	At5g45390	S/T (s)	Clp protease core	Yellow-heart chlorotic appearance (KD)	Zheng et al. 2006
			ClpP5	At1g02560	S/T (s)	Clp protease core	Embryo lethal	Kim et al. 2009
			ClpP6	At1g11750	S/T (s)	Clp protease core	Yellow-heart chlorotic appearance (KD)	Sjögren et al. 2006
			ClpR1	At1g49970	S/T (s)	Clp protease core (no catalytic triad)	Retarded-growth Chlorotic leaves	Kim et al. 2009 Koussevitzky et al. 2007 Stanne et al. 2009
			ClpR2	At1g12410	S/T (s)	Clp protease core (no catalytic triad)	Seedling lethal	Kim et al. 2009
			ClpR3	At1g09130	S/T (s)	Clp protease core (no catalytic triad)	–	–
			ClpR4	At4g17040	S/T (s)	Clp protease core (no catalytic triad)	Seedling lethal	Kim et al. 2009
			ClpT1	At4g25370	S/T (s)	–	–	–
			ClpT2	At4g12060	S/T (s)	–	–	–
			ClpS1	At1g68660	S/T (s)	–	–	–
	SK	S41	CtpA1	At3g57680	T (l)	PDZ domain	High-light sensitive	Yin et al. 2008

(continued)

Table 20.1. (continued)

Protease type	Clan	Family	Protein	Gene name	Location	Protein property	Mutant phenotype	References
	SK	S49	SppA	At1g73990	T (s)	–	Altered responses to high light acclimation	Lensch et al. 2001 Wetzel et al. 2009
	ST	S54	Rhomboid11	At5g25752	–	Intramembrane protease	–	Kimec-Wisniewska et al. 2008
Metallo	MA	M41	**FtsH protease**					
			FtsH1	At1g50250	T (s)	AAA/membrane-bound	No phenotype	Sakamoto et al. 2003
			FtsH2	At2g30950	T (s)	AAA/membrane-bound	Yellow variegated	Chen et al. 2000 Takechi et al. 2000
			FtsH5	At5g42270	T (s)	AAA/membrane-bound	Yellow variegated	Sakamoto et al. 2002
			FtsH6	At5g15250	–	AAA/membrane-bound	No phenotype	Sakamoto et al. 2003 Zelisko et al. 2005
			FtsH7	At3g47060	–	AAA/membrane-bound	–	–
			FtsH8	At1g06430	T (s)	AAA/membrane-bound	No phenotype	Sakamoto et al. 2003
			FtsH9	At5g58870	–	AAA/membrane-bound	–	–
			FtsH11	At5g53170	–	AAA/membrane-bound Dual targeting to mitochondria and chloroplasts	Thermosensitive	Chen et al. 2006
			FtsH12	At1g79560	–	AAA/Membrane-bound	–	–
	ME	M16	SPP	At5g42390	S	–	Most antisense caused lethality to seedlings	Zhong et al. 2003
	ME	M16	PreP1	At3g19170	S	Dual targeting to mitochondria and chloroplasts	Double-knockout mutant shows a chlorotic phenotype	Bhushan et al. 2003, 2005 Moberg et al. 2003 Nilsson et al. 2009
	ME	M16	PreP2	At1g49630	S	Dual targeting to mitochonadria and chloroplasts		
	MM	M50	EGY1	At5g35220	–	Intramembrane protease	Pigmentation-deficient Defective in ethylene-stimulated hypocotyl gravitropism	Chen et al. 2005
	MM	M50	EGY2	At5g05740	–	Intramembrane protease	–	–
	MM	M50	AraSP	At2g32480	IE	Intramembrane protease	Impaired chloroplast development	Bölter et al. 2006

E envelope, *IE* inner envelope, *S* stroma, *T* thylakoid membrane, *s* and *l* stromal and lumenal side of the thylakoid membrane, respectively, *KD* knockdown mutant

[a]Personal communication by L. Zhang and X. Sun

[b]The Clp/HSP100 subunits (ClpC1, ClpC2, ClpB3, and ClpD) of Clp protease complexes are not included in here

degradation pathways of chloroplast proteins and their fine-tuning contribute to nutrient recycling, which is important for proper plant growth and possibly for reproduction as well. In this chapter, we first describe our knowledge of the chloroplast proteolytic machineries in leaf development, and subsequently we describe degradation mechanisms of chloroplast proteins in leaf senescence. For further background informations, we refer the readers to other recent reviews, some of which provide comprehensive information about plastid proteases and their physiological roles in chloroplast homeostasis (Adam 2000; Adam and Clarke 2002; Clarke et al. 2005; Richter et al. 2005; Adam et al. 2006; Sakamoto 2006; Kato and Sakamoto 2009, 2010; Chi et al. 2011; Olinares et al. 2011).

II. Plastid Protein Degradation During Leaf Development

During leaf development, proplastids are converted to mature chloroplasts in a light-dependent manner. Because chloroplast differentiation from proplastids occurs rapidly and dynamically, the synthesis and import of numerous proteins (see also Ling et al., Chap. 12) are necessary for this process. Concomitantly, synthesis of plastid-encoded proteins is activated. Finally the imported proteins from the cytosol and plastid-encoded proteins assemble into various protein complexes coordinately for the proper chloroplast function. Generation of abnormal peptides and proteins is unavoidable during these comprehensive protein dynamics. Therefore, quality control of proteins by proteases is a necessary feature of chloroplast maturation.

A. Proteases Involved in Protein Import

Proteins found in chloroplasts of mature leaves are encoded either by the chloroplast or by the nuclear genome. During differentiation of proplastids to chloroplasts, only a few chloroplast proteins are produced within the organelle; the larger share of chloroplast proteins are synthesized in the cytosol as precursors containing N-terminal transit sequences. These precursors are imported from the cytosol rapidly to developing chloroplasts through the general import machinery called the translocon at the outer envelope membrane of chloroplasts (TOC) and the translocon at the inner envelope membrane of chloroplasts (TIC) (Andres et al. 2010; Kovacs-Bogdan et al. 2010). Mutants lacking a component of the TOC/TIC protein import apparatus frequently show non-photosynthetic albino phenotypes, suggesting the necessity of protein transport into chloroplasts for chloroplast differentiation and plant viability (Andres et al. 2010; Kovacs-Bogdan et al. 2010). After import to the stromal space, their transit peptides are removed by stromal processing peptidase (SPP) (Richter et al. 2005). Originally, SPP was purified from pea as a member of soluble metalloprotease that contains an inverted zinc-binding motif HXXEH at the catalytic site (VanderVere et al. 1995). The cleavage of transit peptides by SPP proceeds in a two-step process. In the first step, SPP binds to the transit peptide of precursors and cleaves it from the mature form. In the second step, the transit peptide is further cleaved by SPP into subfragments and finally released from SPP (Richter et al. 2005). Studies using antisense SPP transgenic plants have elucidated the crucial role of SPP in chloroplast biogenesis. The SPP antisense transgenic tobacco plants display chlorosis and retardation of plant growth (Wan et al. 1998). Similarly, a large share of antisense transgenic plants in Arabidopsis cause seedling lethality (Zhong et al. 2003). Furthermore, the import capacity of precursors is markedly impaired in chloroplasts isolated from the antisense transgenic tobacco; N-terminal transit sequences fused to a reporter Green Fluorescent Protein (GFP) were not transported into chloroplasts but accumulated in the cytosol in these antisense transgenic plants (Zhong et al. 2003). These results verify experimentally that the loss of SPP function affects not only the removal of transit peptides in chloroplasts, but also a series of protein import mechanisms required for chloroplast development.

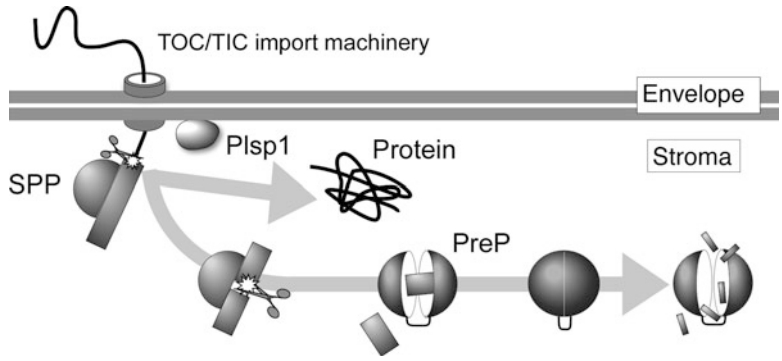

Fig. 20.1. Precursor proteins, imported via TOC/TIC import machinery, undergo processing in the stroma. This figure illustrates removal of transit peptides by peptidases in chloroplasts. To remove transit peptides, SPP binds to the transit peptide and cleaves it from the precursor. Simultaneously, SPP also cleaves the transit peptide into subfragments, which are further degraded by PreP.

Subfragments of transit peptides released from imported precursor proteins are potentially toxic for the integrity of plastid membrane structures and its proper function. Evidence was provided to suggest that the presequence protease (PreP) is responsible for degrading the released subfragments (Ståhl et al. 2005). An ATP-independent metalloprotease, PreP, contains an inverted version of the common zinc-binding motif HXXEH; it has been identified initially as a protease involved in degrading mitochondrial presequences in potato (mitochondrial proteins imported from the cytosol also undergo maturation, and transit peptide sequences of these precursors are termed presequences) (Ståhl et al. 2002). Two homologs, AtPreP1 and AtPreP2, are found in the Arabidopsis genome. Both are dual-targeted to chloroplasts and mitochondria (Bhushan et al. 2003, 2005; Moberg et al. 2003). The unique chamber structure by two halves of this protease connected by a hinge region appears proper for the degradation of the peptide substrates because the chamber size is suitable to hold a small peptide and to exclude larger proteins (Fig. 20.1) (Johnson et al. 2006). Actually, PreP has the capacity to degrade the cleaved precursor peptides that are between 10 and 65 amino acid residues without substrate specificity (Ståhl et al. 2005). Nilsson Cederholm et al. (2009) showed that Arabidopsis double mutants lacking both PreP homologs have a pale green phenotype and retarded plant growth, and, this was due to the presence of aberrant chloroplasts and mitochondria. These observations strongly imply that proper degradation of the subfragments by chloroplast protease is necessary for normal leaf development.

Chloroplast development accompanies biogenesis of thylakoid membranes where photosynthetic light reaction and ATP synthesis occur. Similar to stromal proteins, many proteins in protein complexes of thylakoid membranes and luminal proteins are also synthesized in the cytosol and then transported to other sites. After being moved into the stromal space, proteins targeting the thylakoid lumen are further transported by either the chloroplast secretory (cpSec) or the chloroplast twin-arginine translocation (cpTat) pathway. The proteins that are localized in thylakoid membranes are inserted by the chloroplast signal recognition particle (cpSRP) or via the spontaneous pathway (Cline and Dabney-Smith 2008). To be localized in the thylakoid membrane, many thylakoid proteins have a bipartite transit peptide, which is constituted by a plastid transit peptide and a thylakoid-targeting signal peptide. Proteases functioning for the removal of the thylakoid-targeting signal

peptide are called thylakoidal processing peptidases (TPP). Chaal et al. (1998) demonstrated that membrane-anchored serine-type proteases that belong to the type I signal peptidase (SPase I) family function as TPP. In the Arabidopsis genome, at least three SPase I homologs, plastidic type I signal peptidase (Plsp), exist. In Arabidopsis, Plsp3 was first identified as TPP based on in vitro signal peptidase activity assay (Chaal et al. 1998). Subsequently, Plsp1 has been regarded as a peptidase responsible for maturation of the component of the translocon at the plastid outer envelope membrane: Toc75 (Inoue et al. 2005). Further studies using *plsp1* mutants revealed that Plsp1 also contributes to the processing of several thylakoid luminal proteins (subunits of the oxygen-evolving complex of PSII [OEC33 and OEC23], and plastocyanin) (Shipman and Inoue 2009). These results are consistent with the character of Plsp1 localized not only in the envelope membranes but also in the thylakoid membranes. To date, the in vitro signal peptidase activity of Plsp1 has not been confirmed, but Plsp1 is likely to function as a TPP in vivo. Meanwhile, the result that the lack of Plsp1 caused an abnormal plastid membrane structure and a seedling lethal phenotype suggests, similarity observed in case of SPP, that the the proper processing of thylakoid lumenal proteins is necessary for chloroplast development during leaf growth (Shipman-Roston et al. 2010). Together with studies in SPP and PreP, these observations clearly indicate that a series of protease activities in protein maturation is involved in the early process of chloroplast development.

B. Protein Quality Control During Leaf Development

In addition to the significance of proteases in the protein maturation steps as described above, proteases are well known to play a crucial role in the quality control of proteins in chloroplasts. Presuming that the occurrence of the abnormal proteins that are caused by protein misfolding increases coincidentally with large-scale protein synthesis that is required during chloroplast differentiation, the quality control of proteins is expected to become more important in this step. Actually, the loss of chloroplast proteases often severely impairs chloroplast biogenesis and its homeostasis. In this section, we describe the function of chloroplast proteases that are involved in quality control.

1. Protein Quality Control in Stroma

a. Clp Protease

To date, several proteases have been identified in the chloroplast stroma (Kato and Sakamoto 2010). Among these stromal-localized proteases, Clp is considered to play a central role in quality control as a stromal housekeeping protease (Clarke et al. 2005). Actually, Clp protease is an ATP-dependent serine-type protease complex that is present in almost all bacteria and in mitochondria and chloroplasts (Porankiewicz et al. 1999; Yu and Houry 2007). The functional Clp is constituted of a chaperone that belongs to AAA+ATPases and a proteolytic core component. In *E. coli,* the chaperone components comprise a homogeneous hexameric ring of the Clp/Hsp100 subunits, either ClpA or ClpX containing two and one AAA domains, respectively (Grimaud et al. 1998). The central proteolytic core of the Clp protease in *E. coli* comprises two stacked homogeneous heptameric rings of seven identical ClpP with serine-type proteolytic active sites (Fig. 20.2). These heptameric rings form a barrel-like structure enclosing a large chamber containing exposed proteolytic sites (Wang et al. 1997). The chaperone component docks on one or both ends of the barrel-like core component (Kessel et al. 1995; Grimaud et al. 1998). The connected chaperone components recognize denatured protein substrates; then they unfold and translocate the substrates through the narrow axial pore into the proteolytic chamber. Moreover, a small adapter protein, ClpS, is associated with the Clp chaperone component to prevent accidental degradation of functional proteins

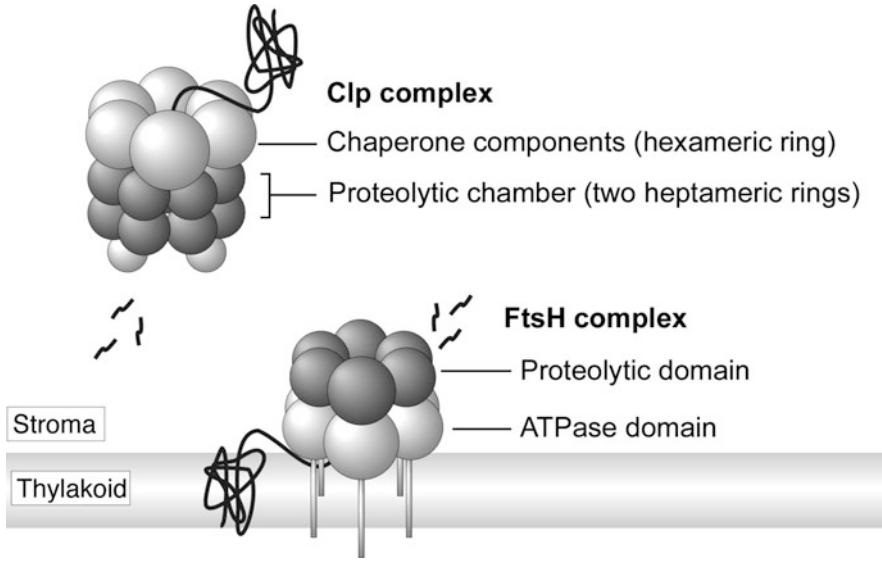

Fig. 20.2. Schematic representation of Clp and FtsH protease complexes in chloroplasts. Clp protease complexes are composed of the catalytic component with two stacked heteroheptameric rings and the chaperone component that comprises a homohexameric ring of the Clp/HSP100 subunits. One of the heteroheptameric rings in the catalytic component contains ClpP1 and ClpR1-4 and the other contains ClpP3-6. The chaperone components are likely to be constituted by ClpC1, ClpC2, and ClpD. In addition, ClpT proteins are associated to the peripheral surface of the proteolytic core complex. On the other hand, FtsH protease complexes constitute heterohexameric ring structure in thylakoid membranes. In contrast to the Clp protease complexes, the subunits of FtsH protease complexes harbor both its proteolytic and ATPase domains in a same polypeptide. Four subunits—FtsH1 and FtsH5 (type A), FtsH2 and FtsH8 (type B)—have been shown to comprise major isomers of chloroplast FtsH complexes.

(Erbse et al. 2006; Wang et al. 2008). These structural features of the Clp protease complex indicate that the Clp complex employs common principles with the 26S proteasome proteolytic machinery in eukaryotes because both Clp and 26S proteasome use the unfolding-coupled processive protein degradation system. Consequently, Clp protease complexes are regarded as the counterparts of eukaryotic proteasomes.

The ClpP subunits are diversified and exist as multiple copies in photosynthetic organisms, in contrast to most bacteria containing a single copy of the proteolytic subunit. In chloroplasts, the proteolytic chamber is present as a heterocomplex; the proteolytic core comprises five ClpP isomers (ClpP1 and ClpP3–P6) and four ClpP-like subunits (ClpR1–ClpR4) that lack the conserved amino acid of the catalytic triad (Peltier et al. 2001, 2004; Clarke et al. 2005; Sjögren et al. 2006). In cyanobacteria, Andersson et al. (2009) have demonstrated that ClpR is indeed proteolytically inactive. Compared with the complexity and the diversity of the proteolytic chamber subunits, the subunits of the chaperone component in chloroplasts are rather simple. Among the four homologs of Clp/Hsp100 subunits (ClpB3, ClpC1, C2, and ClpD) that are localized in chloroplasts (Moore and Keegstra 1993; Weaver et al. 1999; Peltier et al. 2004) with two distinct AAA+domains, three (ClpC1, C2 and ClpD) seem to form a complex with a proteolytic chamber (Peltier et al. 2004). In addition, a homolog of an adapter protein ClpS has been discovered in chloroplast Clp complexes. Along with these subunits, two novel plant-specific subunits, ClpT1 and ClpT2, which share homology with the N-terminal domain of Clp/Hsp100 proteins, have been identified (Peltier et al. 2001, 2004). Further studies showed that the

peripheral attachment of ClpT proteins to the proteolytic core components is likely to regulate the assembly of Clp protease complex (Sjögren and Clarke 2011).

The physiological importance of the Clp protease was initially demonstrated by studies showing that tobacco transgenic lines with significantly reduced levels of ClpC or ClpP1 could not survive (Shanklin et al. 1995; Shikanai et al. 2001). Additional studies using Arabidopsis mutants and antisense lines supported the results of early studies and revealed the indispensable function of the Clp protease (Rudella et al. 2006; Sjögren et al. 2006; Kim et al. 2009; Stanne et al. 2009; Zybailov et al. 2009). Knockout mutants showed that the defect of the ClpP subunit in protease complexes engenders a lethal phenotype in Arabidopsis. Therefore, the antisense strategy was employed for additional analysis of ClpP. The ClpP4 and P6 antisense plants showed leaf chlorosis and slow growth (Sjögren et al. 2006; Zheng et al. 2006). In particular, the phenotypes of mutants are severe in younger inner leaves. This impairment of chloroplast development by repression of Clp suggests a functional importance of Clp in the early phase of plastid differentiation. Of the *clpr* mutants, the *clpr1* null mutant can survive because the ClpR1 function is partially complemented by the closely related ClpR3 protein (Koussevitzky et al. 2007; Kim et al. 2009; Stanne et al. 2009). The loss of ClpR1 brings about leaf chlorosis and reduces photosynthesis rates; further, the more severe phenotype of the *clpr1* mutant in the younger leaves suggests the involvement of Clp function in chloroplast development. The proteomic analysis using isolated chloroplasts from ClpP6 antisense and *clpr1* mutant revealed some potential substrates for Clp protease, which are involved in general homeostatic roles such as protein synthesis, protein maturation, and RNA maturation (Sjögren et al. 2006; Stanne et al. 2009). Furthermore, large-scale comparative proteomics, using the null-mutant *clpr4* shows albino phenotype, that cannot survive beyond the seedling stage, but is able to grow under heterotrophic conditions, demonstrated that many chloroplast proteins had changed in the *clpr4* mutant (Kim et al. 2009). Especially, the nearly complete loss of photosynthetic proteins in the mutant is thought to be the reason for the severe phenotype in *clpr4*. Similarly, the large-scale comparative proteomics of the *clpr2* knockdown mutant and the wild-type showed that impaired protease activities are involved widely in various chloroplast protein accumulation including the methylerythritol phosphate (MEP) pathway proteins, which have been implicated in other reports as substrates of Clp protease (Zybailov et al. 2009). In addition, the loss of function of chaperone component subunit ClpC1 causes growth retardation and leaf chlorosis with reduced photosynthesis proteins (Constan et al. 2004; Sjögren et al. 2004; Kovacheva et al. 2005). Moreover, the lack of both ClpC1 and ClpC2 causes very early embryo lethality (Kovacheva et al. 2007). These results suggest important roles of the unfolding function of the Clp protease complex for plastid development and plant viability. Collectively, the studies of the Clp protease indicate that the derangement of protein degradation resulting from impairment of Clp causes the disruption of chloroplast development with disintegration of protein homeostasis.

b. Protein Degradation and Chaperones

We note that the loss of Clp subunits, not only Clp/Hsp100 subunits that are likely to function as a chaperone independently, but also Clp protease core subunits, causes accumulation of molecular chaperones (unfoldase ClpB3, chloroplast Hsp90, Hsp70, Cpn60 and so on) (Kim et al. 2009; Stanne et al. 2009; Zybailov et al. 2009). The molecular chaperones help in proper protein folding to prevent the generation of harmful polypeptides and aggregation of proteins. Apparently, the higher level of molecular chaperones results from the increased protein instability, which appears to be associated with the accumulation of undegraded

harmful polypeptides in *clp* mutants. Therefore, the increased level of chaperones in *clp* mutants suggest the presence of a close relation between protein degradation and the folding for protein quality control in chloroplast protein synthesis and protein transport from the cytosol. To date, many research groups have identified molecular chaperones in chloroplasts; many reviews describe their functions (Jackson-Constan et al. 2001; Levy-Rimler et al. 2002; Weiss et al. 2009). Here, we briefly explain their roles and involvement in chloroplast development.

One chloroplast Clp/Hsp100 subunit, ClpB3, seems to function as a chaperone independently of Clp protease complexes because it lacks the conserved domain for binding to ClpP (Clarke et al. 2005). The mutant of ClpB3 is reported as one of the *albino* or *pale-green* (*apg*) mutants, *apg6*, and it shows severe defects in chloroplast development (Myouga et al. 2006; Lee et al. 2007). Further, ClpC1 and ClpC2, also known as Hsp93-V and Hsp93-III, have been identified as proteins associated with the chloroplast protein import apparatus (Akita et al. 1997; Nielsen et al. 1997). Because a large proportion of ClpC is present in the stroma in a soluble form, the primary function of ClpC is thought to serve as the chaperone component of the Clp protease complexes; however, a role as an independent chaperone in chloroplast protein import also seems feasible. Another Hsp protein, chloroplast Hsp90, was originally identified through a chlorate-resistant mutant that shows an altered response to red light (Cao et al. 2003). The mutant of chloroplast Hsp90 also shows delayed chloroplast development, suggesting the involvement of chloroplast Hsp90 in the maturation of newly imported or synthesized proteins during chloroplast development (Cao et al. 2003). Regarding Hsp70 homologs, four Hsp70 systems have been detected within the different compartments of chloroplasts (Jackson-Constan et al. 2001). Two of them, Com70 and IAP70, both localized in the envelope membrane, are apparently involved in protein import to chloroplasts (Schnell et al. 1994; Kourtz and Ko 1997). The other two Hsp70, cpHsc70-1 and cpHsc70-2, which have been shown to accumulate in *clp* mutants, are apparently present in stroma (Su and Li 2008). An Arabidopsis T-DNA insertion mutant of cpHsc70-1 shows variegated cotyledons and malformed leaves, although a T-DNA mutant of cpHsc70-2 shows no visible phenotype (Su and Li 2008). Furthermore, attempts to obtain a double mutant and the approach of co-suppression of both genes using RNAi interference showed their redundant functions and their important role in chloroplast development and plant viability (Latijnhouwers et al. 2010). In addition to these homologs, the homologs of the bacterial chaperone GroEL, Cpn60s, are reported to be proteins that accumulate in *clp* mutants. Similar to Hsp70s, Cpn60, which interacts with a component of the inner membrane import apparatus Tic110, helps the imported protein to fold into its native conformation (Kessler and Blobel 1996). The results demonstrating that the *cpn60* mutants show abnormal development of embryos suggest the necessity of Cpn60 for plastid biogenesis and plant viability (Apuya et al. 2001). In addition to these typical molecular chaperones, the chaperone activity of Deg protease was recently reported as described below.

2. Protein Quality Control in Membranes

a. FtsH Protease

The quality control of membrane proteins is necessary for chloroplast biogenesis, especially in the formation of thylakoid membranes. Of the membrane proteases, FtsH protease is the best characterized one because the unique variegated phenotype of the *ftsh* mutants has been the subject of interest of many studies. FtsH protease is a membrane-bound ATP-dependent metalloprotease that was originally identified in *E. coli* as a necessary protein for growth (Tomoyasu et al. 1993). FtsH is a large complex that has a AAA+ATPases domain and a proteolytic core component (Krzywda et al. 2002; Niwa et al. 2002); however, in contrast to

the Clp protease complex, it has both domains on the same polypeptide chain (Fig. 20.2). Crystal structure studies have revealed that six identical FtsH subunits form a large complex with unique catalytic site(s) located at the peripheral region of the hexamer ring (Bieniossek et al. 2006; Suno et al. 2006).

We note that FtsH is highly conserved in all organisms. In plants, FtsH homologs have been isolated and characterized from, e.g., spinach, tobacco and peas (Lindahl et al. 1996; Seo et al. 2000; Yue et al. 2010). In Arabidopsis, among the enumerated 12 homologous genes, 9 homologs (FtsH1, 2, 5, 6, 7, 8, 9, 11, and 12) are located in the chloroplast (Sakamoto et al. 2003). Of these homologs, FtsH1, 2, 5, and 8 are identified by immunoblot and proteomic analyses of the isolated thylakoid membrane (Sakamoto et al. 2003; Friso et al. 2004; Sinvany-Villalobo et al. 2004; Yu et al. 2004). Immunoblot analysis showed that the proteolytic domain faces the stroma side of the membrane (Lindahl et al. 1996; Sakamoto et al. 2003). The four isomers, mentioned above, have been regarded as major isomers of chloroplast FtsH complexes. They are divided into closely related pairs of two types, FtsH1 and FtsH5 (type A), FtsH2 and FtsH8 (type B), which are likely duplicated (Sakamoto et al. 2003). Among them, FtsH2 is the most abundant isomer, followed by FtsH5, FtsH8, and FtsH1. Originally, FtsH2 and FtsH5 were reported as responsible genes of leaf-variegated mutants, which have long been known in Arabidopsis (Chen et al. 2000; Takechi et al. 2000; Sakamoto et al. 2002). The mutants of FtsH2 and FtsH5 are called *yellow variegated 2* (*var2*) and *var1*; *var2* shows severe variegated phenotype and *var1* shows weak leaf-variegation. On the other hand, mutants with a loss of FtsH1 and FtsH8 show no visible change in their phenotype. The difference in the degree of leaf-variegation among these mutants is considered to be dependent on the abundance of isomers in the FtsH heterocomplexes. The variegated phenotypes in *var1* and *var2* mutants are rescued, respectively, by overexpression of FtsH1 and FtsH8; this suggests functional redundancy of each type A and type B FtsH isomers (Yu et al. 2004, 2005). Furthermore, the results that *ftsh2 ftsh8* and *ftsh1 ftsh5* double mutants showing an albino-like phenotype underscore the possibility that the existence of both type A and type B isomers is required for the proper function of FtsH (Zaltsman et al. 2005b). Together with the studies by proteomics and the biochemical approaches, the construction of heterohexameric FtsH complex by type A and type B isomers in chloroplasts has been published.

Additionally, the observations that overall FtsH levels correlate with the degree of white sectors in leaves led to the proposal of a threshold model of leaf variegation; subthreshold amounts of the FtsH complex block thylakoid formation in leaf development, leading to the failure of chloroplast development (Yu et al. 2004). In this threshold model, it was expected that FtsH would have a decisive role at a particular stage of chloroplast development. The result that white viable sectors of *var2* mutants have undifferentiated plastids, and the observations that the variegated pattern is irreversible once developed, supports this expectation (Zaltsman et al. 2005a; Kato et al. 2007). Furthermore, recent careful observation of plastid ultrastructures during the early stage of leaf development demonstrated that the abnormal plastids in white sectors is formed as a result of the arrest of chloroplast development at its initial steps (Sakamoto et al. 2009). To summarize, these results demonstrate that the early stage of chloroplast development requires sufficient levels of FtsH for thylakoid formation.

Further studies have shown that several genetic defects cause the suppression of leaf-variegation. Most identified mutant genes are involved in the chloroplast translation (chloroplast rRNA processing and protein synthesis), which implies that the delay in chloroplast development, which results from the impairment of chloroplast translation, lowers the requirement of FtsH for protein quality control in chloroplast biogenesis (Park and Rodermel 2004; Miura et al. 2007;

Yu et al. 2008; Liu et al. 2010). Electron-microscopic observations of plastids revealed that the loss of FtsH in *var2* mutant causes slow progression of chloroplast development in the prospective green sectors (Sakamoto et al. 2009). In other words, the variegated mutant seems to avoid the serious dysfunction of chloroplasts by limiting the progression of chloroplast differentiation in early leaf development. Taken together, these results indicate that the balance between the speed of thylakoid development and the protein quality control by FtsH is an important point for proper chloroplast development and that it is regulated strictly by communication between the nucleus and the chloroplasts.

Using transgenic plants that ectopically expressed a proteolytically inactive FtsH2, Zhang et al. (2010) posed an interesting question for the FtsH function in leaf variegation; the result that expression of proteolytically inactive FtsH2 rescued not only leaf variegation in *var2*, but also seedling lethality in *ftsh2 ftsh8* double mutant suggests that not all proteolytic activities of FtsH heterocomplexes are necessary for their function in chloroplasts (Zhang et al. 2010). Further analysis of *var1 var2* mutants, with expression of proteolytically inactive FtsH2, and a study using an inducible FtsH2, show that the overall amount of FtsH complexes predominantly determines the threshold of chloroplast development when the protease activity is excessive (Zhang et al. 2010).

What is the substrate of FtsH in chloroplast development? Using mature chloroplasts, several studies suggest only a partial answer to this question. In vitro studies showed the involvement of FtsH in the degradation of unassembled Rieske Fe-S protein and in the degradation fragment of the PSII reaction center D1 protein (Ostersetzer and Adam 1997; Lindahl et al. 2000). Additional in vivo analyses using mutants lacking FtsH2 or FtsH5 also indicated the involvement of FtsH in D1 degradation (Bailey et al. 2002; Kato et al. 2009). Most of the available evidence point to the central role of FtsH in D1 degradation in the PSII repair cycle in mature chloroplasts. However, the study of the substrates of FtsH in the chloroplast developmental stage has scarcely been made. Consequently, although D1 protein might be a possible substrate in developing thylakoids as well as that in mature chloroplasts, the substrates of FtsH in the chloroplast developmental stage remain largely unknown.

b. Deg Protease

DegP protease is an ATP-independent serine type protease that was originally identified in *E. coli* as the protease necessary for survival at high temperatures (Skorko-Glonek et al. 1995). It has two domains: the proteolytic domain at the N-terminus and the PDZ domain(s), which are involved in protein–protein interactions, at the C-terminus (Clausen et al. 2002). It is noteworthy that DegP has not only a proteolytic activity, but also a chaperone activity to prevent the accumulation of abnormal proteins (Spiess et al. 1999). The switch between protease and chaperone function seems be regulated by a conformation change of the protein caused by a temperature shift. The chaperone activity is predominant at low temperatures; protease activity is increased at elevated temperatures (Spiess et al. 1999). In Arabidopsis, among the 16 DegP homologous genes, five homologs (Deg1, 2, 5, 7, and 8) have already been found in the chloroplast (Itzhaki et al. 1998; Haussühl et al. 2001; Peltier et al. 2002; Schubert et al. 2002; Huesgen et al. 2005; Sun et al. 2007, 2010a). Furthermore, the presence of Deg11 in the chloroplast stroma has also been suggested (L. Zhang and X. Sun, personal communication, 2011). These homologs are generally peripherally attached to the stromal (Deg2 and 7) or the lumenal sides (Deg1, 5, and 8) of thylakoid membranes. In *E. coli* DegP peripherally attaches to the plasma membrane. Early in vitro studies using recombinant proteins showed that Deg1 proteases are involved in the degradation of several thylakoid lumen proteins, such as plastocyanin and OEC33 (Chassin et al. 2002), and Deg2 functions in the initial endoproteolytic cleavage of the D1 protein

(Haussühl et al. 2001), although the in vivo contributions of these Deg proteases to the degradation of these proteins remain unconfirmed (Huesgen et al. 2006). Meanwhile, there is evidence that several Deg proteases (Deg1, 5, 7, and 8) play an important role in the in vivo PSII repair under high-light conditions (Sun et al. 2007, 2010a, b). The work of Kapri-Pardes et al. (2007) suggests the importance of Deg protease for plant viability since the authors failed to obtain homozygous knockout lines of Deg1. Furthermore, antisense lines with a reduced level of the Deg1 protein showed pale-green phenotypes, suggesting the requirement of Deg1 for chloroplast biogenesis and homeostasis (Kapri-Pardes et al. 2007). It is particularly interesting that Sun et al. (2010b) show the existence of chaperone activity of Deg1, like it is known for *E. coli* homolog DegP. However, the functions of Deg1 in chloroplast development are largely unknown, although the chaperone activity of Deg1 seems to be very important for proper protein assembly that is needed in thylakoid development.

c. Intramembrane Proteases

Chloroplasts have several intramembrane proteases to degrade membrane proteins for proper chloroplast biogenesis. Of these intramembrane proteases, the loss of the homologs of the sterol-regulatory element binding protein site 2 protease (SREBP S2P protease) causes the impairment of chloroplast biogenesis. One homolog of SREBP S2P proteases, ethylene-dependent gravitopism-deficient and yellow-green 1 (EGY1), is an ATP-independent metalloprotease with eight putative transmembrane helices in its C-terminus (Chen et al. 2005). The *egy1* mutant was originally identified as a mutant that shows abnormal gravicurvature in hypocotyls and a pale-yellow phenotype. The development of the thylakoid membrane system is severely impaired in the mutants. In particular, the remarkable decrease of the levels of the chlorophyll-binding proteins in the *egy1* mutants seems to cause the loss of grana stacks because it is considered that the LHCII-mediated physical connection contributes to the stability of grana stacking (Chen et al. 2005). The association of EGY1 with chloroplast membranes has been confirmed by immunoblotting analysis, but the detailed localization of EGY1 in chloroplasts has not yet been identified. However, another homolog of the SREBP S2P proteases, AraSP, is localized in the chloroplast inner envelope membrane (Bölter et al. 2006). The deduced amino acid sequence of AraSP indicates 4–5 transmembrane helices and a conserved catalytic motif, which is localized between the first two helices. The T-DNA knockout mutant of AraSP cannot germinate and the heterozygous T-DNA insertion mutant still shows severely impaired plant growth (Bölter et al. 2006). Together with the defective phenotype of chloroplast biogenesis in the AraSP antisense lines, these results underscore the important role of AraSP during leaf development (Bölter et al. 2006).

III. Plastid Protein Degradation During Leaf Senescence

In response to organ developmental status and various environmental signals, chloroplasts change their morphology and convert the plastid type from one form to another. This happens also in the final stage of leaf development, when photosynthesis is no longer required, but senescence is initiated. Chloroplasts in senescing leaves gradually shrink and transform themselves into gerontoplasts (Wise 2007). Breakdown of thylakoid membranes, degradation of photosynthetic proteins, and accumulation of a remarkable number of plastglobuli occur during leaf senescence (Krupinska 2007). Although the chloroplast-to-gerontoplast transition occurs in senescing leaves, it is an extremely important event for plant growth because the chloroplast nutrients that are generated by degradation of accumulated proteins during leaf senescence contribute to crop yields and biomass accumulation (Mae 2004).

Despite numerous studies on the degradation of chloroplast proteins and the change of chloroplast structure during senescence, the fate of chloroplasts and the degradation mechanism of chloroplast proteins during senescence remain poorly understood. In the following sections, degradation processes of chloroplast proteins mediated by the proteases are summarized. For additional information, we refer the readers to several reviews on chloroplast component degradation during leaf senescence (Hortensteiner and Feller 2002; Feller et al. 2008; Gregersen et al. 2008; Martinez et al. 2008b).

A. Degradation of Stromal Proteins

Available results suggest that different mechanisms exist for chloroplast protein degradation during leaf senescence (Minamikawa et al. 2001; Chiba et al. 2003; Kato et al. 2004; Otegui et al. 2005). Chloroplast protein degradation mechanisms may be divided broadly into two categories: the degradation of proteins inside the chloroplasts and the degradation of chloroplast proteins in other organelles such as lytic vacuoles (see also Wada and Ishida, Chap. 19). Chloroplast protein degradation pathways that interact with other organelles can be divided further into at least three categories: degradation of chloroplast proteins via chloroplast-derived vesicles, the degradation of engulfed whole-chloroplasts within the vacuole, and the degradation of chloroplasts as a result of tonoplast rupture. It is particularly interesting that studies of Arabidopsis mutants show multiple degradation pathways of chloroplast stromal proteins and their fine-tuning (Hortensteiner and Feller 2002; Feller et al. 2008; Gregersen et al. 2008; Martinez et al. 2008b). We provide below an overview of stromal protein degradation.

1. Protein Degradation Inside Plastids

The idea that proteins are degraded inside chloroplasts is supported by the finding that the protein level decreases rapidly in the early phase of leaf senescence prior to the decline of the chloroplast number (Friedrich and Huffaker 1980; Mae et al. 1984). In particular, stromal enzymes, known to be involved in carbon and nitrogen assimilation, such as glutamine synthetase and ribulose 1, 5-bisphosphate carboxylase-oxygenase (Rubisco), are lost in the early phases of leaf senescence. During this process, soluble proteins are gradually degraded as leaves age; then degraded products are exported to reproductive organs to salvage nutrients. Furthermore, experiments using isolated chloroplasts, which exclude contamination of proteases derived from other cellular components, showed that hydrolysis of Rubisco takes place inside the isolated organelles (Ragster and Chrispeels 1981; Mitsuhashi and Feller 1992).

Although the importance of proteolysis inside chloroplasts has been recognized, plastid proteases involved in protein degradation in senescent leaves remain poorly understood. Early biochemical approaches for the identification of plastid proteases, involved in degradation of Rubisco, suggest that a stromal metalloprotease, that has been partially purified from pea chloroplasts, is able to degrade the large subunit of isolated Rubisco to smaller polypeptides in vitro (Bushnell et al. 1993). A further study using isolated chloroplasts also showed that metalloprotease activities are involved in stromal protein degradation under dark or nitrogen-starvation conditions (Roulin and Feller 1998). However, the metalloprotease that actually functions in chloroplast protein degradation in vivo during leaf senescence remains unidentified.

Various proteases localized in chloroplasts are known to participate in proper organellar functioning through protein quality control. Of these chloroplast proteases, Clp and Lon are considered to participate in protein degradation in the stroma, and FtsH and Deg are known to be involved in protein degradation in thylakoid membranes (Kato and Sakamoto 2010). Of these proteases, one Clp chaperone subunit, ClpD, was first reported to show early response to dehydration (ERDs) at the mRNA level (Kiyosue et al. 1993); ClpD/ERD corresponds to

SAG15 (senescence-associated gene 15), whose transcripts have been known to accumulate during leaf senescence (Nakashima et al. 1997). Although several research groups have reported up-regulation of the mRNA levels of *clpD* during leaf senescence, the ClpD protein does not show any significant accumulation; instead, it declines during senescence (Nakabayashi et al. 1999; Weaver et al. 1999). In spite of up-regulation of genes corresponding to several other subunits of the Clp protease core complex (ClpP3 and ClpP5) has been reported, ClpP protein levels remained almost constant or declined during leaf senescence (Nakabayashi et al. 1999). Consequently, further research is necessary to examine if Clp participates in the degradation of stromal proteins during leaf senescence.

It is noteworthy that the aspartic protease CND41 has been localized in plastids and shown to be up-regulated in natural leaf senescence (Kato et al. 2004). In contrast to Clp, its up-regulation has been detected at both transcriptional and protein levels. This CND41 was originally isolated from chloroplast nucleoids, a large complex of chloroplast DNA and proteins in photomixotrophically cultured tobacco cells (Nakano et al. 1997). Unlike many other major chloroplastic proteases, CND41 that belongs to A1 aspartic protease family (pepsin-like family), appears to be of eukaryotic origin. Studies on the proteolytic activity of CND41, that had been purified from cultured tobacco cells, showed that CND41 can degrade denatured inactive Rubisco at physiological pH (Murakami et al. 2000). However, native active Rubisco appeared to be a poor substrate for it. Furthermore, characterization of transgenic tobacco lines overexpressing CND41 implied that there is a post-translational activation mechanism for CND41 to degrade chloroplast proteins during leaf senescence in vivo (Kato et al. 2005). Aside from tobacco, up-regulation of CND41 homologs during senescence has been observed in Arabidopsis and barley (Parrott et al. 2007; Diaz et al. 2008). It is particularly interesting that immunoblot analysis of Arabidopsis recombinant inbred lines, which were selected based on the differential leaf senescence phenotypes, revealed that the CND41 homolog appeared to accumulate in early senescing Arabidopsis lines, suggesting that the CND41 homolog is associated with senescence (Diaz et al. 2008). These results imply that CND41 contributes to protein degradation inside chloroplasts during leaf senescence in many plant species.

2. Protein Degradation in Other Organelles

The chloroplast number per cell decreases during leaf senescence (Ono et al. 1995; Inada et al. 1998; see also Mulisch and Krupinska, Chap. 14). This decrease was suggested to indicate the presence of a whole chloroplast degradation system, possibly conducted by other organelles. Furthermore studies revealed the presence of small spherical bodies containing chloroplast stromal proteins during leaf senescence (Chiba et al. 2003; Martinez et al. 2008a). We summarize below the degradation pathways of chloroplast proteins via chloroplast-derived vesicles and the degradation of engulfed whole-chloroplasts within the vacuole (see also Wada and Ishida, Chap. 19). These degradation pathways participate in leaf senescence, although chloroplast protein degradation after tonoplast rupture seems to occur mainly during programmed cell death.

a. Rubisco-Containing Bodies

Immunocytochemical detection of Rubisco in naturally senescing wheat leaves showed the existence of distinct small spherical bodies, termed Rubisco-containing bodies (RCBs), in the cytoplasm and in the vacuole (Chiba et al. 2003). In fact, RCB-like structures have been observed in senescent leaves of tobacco (Prins et al. 2008). They also contain another stromal protein, glutamine synthetase, but do not include thylakoid membrane proteins and chlorophylls. The RCBs are 0.4–1.2 μm in diameter and are surrounded by double membranes. Careful observation of RCBs revealed that they are

further surrounded by the other membrane structures, suggesting that an autophagy mechanism is involved in degrading chloroplast proteins (Chiba et al. 2003). Arabidopsis mutants that are defective in autophagy-related genes (*atg* mutant) were recently examined for the relevance of autophagy for RCB formation (Ishida et al. 2008). The analysis of visualized RCBs using GFP-labeled Rubisco shows that RCBs are not observed in leaves of *atg* mutant, although RCBs are observed in the lumen of the vacuoles in the wild-type. Furthermore, characterization of a GFP-linked ATG protein as an autophagy marker demonstrated that the GFP signals are co-localized in autophagic bodies with chloroplast stroma-targeted DsRed. These results show that transfer of stromal proteins to the central vacuole via RCBs pathway requires ATG-dependent autophagy. Another study using an *atg* mutant showed that the size of chloroplasts did not decrease in the *atg* mutant during leaf senescence, suggesting that ATG-dependent autophagy mediated degradation of chloroplast proteins is responsible for chloroplast shrinkage in the senescent stage (Wada et al. 2009). It is particularly interesting that the defect in the generation of RCBs in the *atg* mutant induced the increase of stromules as compared to the stromules in the wild-type (Ishida et al. 2008). The release of small vesicles from the ends of stromules has been observed, and the vesicle diameter is similar to that of RCBs (Gunning 2005). These observations imply the possible association between the release of small bodies from stromules and RCBs.

b. Senescence-Associated Vacuoles

Senescence-associated vacuoles (SAVs) are another type of vesicles that were originally identified in the senescent leaves of soybean and Arabidopsis using the SAG12-GFP fusion protein as a fluorescent marker (Otegui et al. 2005; see also Costa et al., Chap. 18). Distinguishable from the central vacuole with respect to their acidic pH, SAVs have intense proteolytic activity. A typical SAV is approximately 0.7 μm in diameter. In addition, the senescence specific cysteine protease SAG12 is localized selectively within SAVs. Despite the strong relationship of SAG12 and senescence, however, the Arabidopsis *sag12* mutant lacking SAG12 shows no altered senescence phenotypes and has SAVs, indicating that SAG12 is not directly required for SAV formation (Otegui et al. 2005). Direct evidence of the involvement of SAVs in chloroplast protein degradation was obtained by using tobacco transformants expressing chloroplast-targeted GFP (Martinez et al. 2008a). During leaf senescence, GFP that is targeted to the chloroplast stroma was relocalized to SAVs. Furthermore, apart from GFP, Rubisco and glutamine synthetase are contained in isolated SAVs and are degraded within SAVs. Although the D1 protein of the photosystem II reaction center and the light-harvesting complex II (LHC-II) are not contained in SAVs, some SAVs with chlorophyll autofluorescence have been detected (Martinez et al. 2008a). The SAVs are known to be surrounded by a single membrane in contrast to RCBs that have double membranes, but there are several known similarities between RCBs and SAVs. Although the possible relation between these two types of vesicles is currently unknown, SAVs appear to be formed in *atg7* (Otegui et al. 2005), which is defective in the ATG-gene-dependent autophagy and the formation of RCB. The selectivity of proteins that are carried in these compartments as substrates, is still an unanswered question.

c. Degradation Inside the Central Vacuole

In addition to the above-mentioned specific vesicles associated with leaf senescence, the central vacuole, the largest lytic compartment in mature cells, is an important compartment related to chloroplast degradation. An early study using electron microscopy showed the possible physical interaction between the outer envelope of the chloroplast and the tonoplast (Peoples et al. 1980); in this report, the outer envelope of some chloroplasts flanked by the tonoplast was apparently degraded and merged with the tonoplast, but the inner envelope of the chloroplasts appeared to

remain intact. However, another ultrastructural study showed that some chloroplasts observed in mesophyll cells appeared to move toward the center of the cell as leaf senescence proceeded (Wittenbach et al. 1982). This movement was concomitant with the decrease of chlorophylls and soluble proteins and the decline of chloroplast number per cell, suggesting degradation of engulfed whole-chloroplasts within the vacuole. These observations have been supported by an electron microscopic examination of dark-induced senescence of French bean leaves (Minamikawa et al. 2001). Electron microscopic observations indicates chloroplast internalization into vacuoles, and the disruption of the outer membranes of chloroplasts in vacuoles. In this study, the possible involvement of vacuolar cysteine proteases in degradation of chloroplast proteins was suggested. Although the debate about the degradation of proteins in vacuoles and the uptake of chloroplasts by vacuoles under natural leaf senescence continues, it seems that the degradation of whole chloroplasts inside vacuoles is a key step for chloroplast protein degradation. Apparently, the question arises of how chloroplasts are transferred into vacuoles. Wada et al. (2009) have provided a novel finding related to this question: Their observations showed that chloroplasts were found within some vacuoles that were isolated from individually darkened leaves of the wild type, but no chloroplasts were found in the vacuole of an *atg* mutant. Furthermore, the decrease in the chloroplast population was inhibited in *atg* mutant, whereas the loss of chlorophylls and the decrease of soluble proteins in the mutant were comparable to those in the wild-type. These results demonstrate that autophagy also plays a key role in the transport of whole chloroplasts into vacuoles during leaf senescence.

B. Degradation of Thylakoid Membrane Proteins

Thylakoid membranes contain multiple protein complexes (e.g., Photosystem I, Photosystem II, the cytochrome b_6f complex and the light-harvesting complex), which play an important role in light-harvesting and the light-dependent reactions of photosynthesis (see Joshi et al., Chap. 28 in this book). More than 30% of the chloroplast proteins are in the protein complexes of the thylakoid membranes. Therefore, the proteins in these complexes are also considered important as nutrient sources during leaf senescence (Krupinska 2007). During leaf senescence, the structure of thylakoid membranes changes dramatically. The progressive loss of grana stacking, disappearance of thylakoid membranes and massive accumulation of plastoglobuli occur at this stage (Krupinska 2007; see also Mulisch and Krupinska, Chap. 14). These structural changes are caused by the massive degradation of the protein complexes and the lipids in the thylakoid membranes. In this section, we describe the current knowledge of the degradation mechanisms of proteins in the thylakoid membranes and the participation of proteases during leaf senescence.

1. Degradation Mechanisms of Thylakoid Proteins

Of the chloroplast-protein degradation pathways described above, RCBs do not exhibit chlorophyll autofluorescence and SAVs do not contain thylakoid proteins, whereas chlorophyll *a* was detected in isolated SAVs (Ishida et al. 2008; Martinez et al. 2008a; see also Costa et al., Chap. 18). Consequently, the degradation of proteins inside chloroplasts and the uptake of chloroplasts by vacuoles are expected to become more important in the degradation of thylakoid membrane proteins. Of thylakoid membrane protein complexes, most thylakoid membrane proteins appear to bind chlorophylls and carotenoids. Accumulating evidence points to fine-tuned regulation between the breakdown of chlorophylls and degradation of apoproteins during leaf senescence (Hörtensteiner, Chap. 16). Loss of chlorophyll *b* reductase in Arabidopsis (NYC1 and NOL), which catalyzes the first step of chlorophyll *b* degradation, results in a non-functional *stay-green* phenotype that impairs chlorophyll catabolism but shows other senescence processes (Kusaba et al. 2007; Sato et al. 2009). In addition,

pheophorbide *a* oxygenase (PAO) and pheophytin pheophorbide hydrolase (PPH) deficiencies result in a non-functional stay-green phenotype (Pruzinska et al. 2003; Schelbert et al. 2009). These stay-green mutants, which retain chlorophylls in senescent leaves, show highly stable LHC proteins, suggesting the requirement of chlorophyll degradation for the full degradation of thylakoid membrane proteins, especially LHC proteins. These findings indicate a close relation between chlorophylls and apoproteins and suggest that the degradation of thylakoid membrane proteins is strictly controlled. Because free chlorophyll is potentially phototoxic, the degradation of chlorophylls during the degradation of thylakoid membrane proteins needs to be tightly controlled. In spite of rapid progress made towards the understanding of chlorophyll degradation (see Hörtensteiner, Chap. 16 in this book), elucidation of the degradation mechanism of thylakoid membrane proteins is lagging.

2. Proteases Involved in Degradation of Membrane Proteins

The best characterized protein-degradation mechanism in thylakoid membranes is the degradation of the PSII reaction center D1 protein in the PSII repair cycle. In higher plants, as described above, much evidence indicates that the thylakoid membrane-bound FtsH and Deg proteases play crucial roles in this process (Kato and Sakamoto 2009). Meanwhile, a study of the Clp protease suggested the cytochrome b_6f complex to be a putative substrate for Clp during nitrogen starvation in the green alga *Chlamydomonas reinhardtii* (Majeran et al. 2000). Lon protease, an ATP-dependent serine protease, and SppA protease, an ATP-independent serine protease, are closely associated with the stromal side of thylakoid membranes (Lensch et al. 2001; Ostersetzer et al. 2007). These proteases also seem to play roles in proteolysis of the thylakoid membrane (Wetzel et al. 2009), although their substrates are poorly understood. These reports specifically address protease functions in the mechanism of protein homeostasis, but do not explain the role of the protease during leaf senescence. We hope that further studies will reveal the role of the major chloroplast proteases in the degradation of thylakoid membrane proteins in leaf senescence.

IV. Concluding Remarks

Over the past two decades, much effort has been devoted to identify and characterize chloroplast proteases. Consequently, numerous chloroplast proteases that are homologous with known bacterial proteases have been identified. The analyses of Arabidopsis mutants has revealed, as described in this chapter, that some are necessary not only for chloroplast biogenesis but also for plant viability. However, the specific substrates of these proteases during leaf development remain unclear. Exploring the recognition mechanisms of substrates in these proteases constitutes an important area of future studies in this field. Additionally, communications between proteolytic activities and chloroplast development, like the delayed chloroplast development that is observed in the variegated mutants, is expected to become an interesting area of investigation. Our knowledge about chloroplast protein degradation during leaf senescence remains poor despite recent efforts in unravelling the mechanisms of protein degradation. The difficulty in elucidation of the mechanism of protein degradation during leaf senescence is apparently due to the complexity of leaf senescence that results from the involvement of multiple factors (e.g., light, nutrition, and the developmental stage of the plant). The contribution of chloroplast proteases in protein degradation during leaf senescence is an important question that should be examined critically in future studies. We hope that greater efforts in this field will answer these questions.

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