Protein Degradation Machineries in Plastids

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Key Words
protease, ATP-dependent proteolysis, plastid and chloroplast, photosynthesis and oxidative damage, thylakoid membranes

Abstract
Plastids undergo drastic morphological and physiological changes under different developmental stages and in response to environmental conditions. A key to accomplishing these transitions and maintaining homeostasis is the quality and quantity control of many plastid proteins by proteases and chaperones. Although a limited number of plastid proteases have been identified by biochemical approaches, recent progress in genome information revealed various plant proteases that are of prokaryotic origin and that are localized in chloroplasts. Of these, ATP-dependent proteases such as Clp, FtsH, and Lon are considered the major enzymes involved in processive degradation (gradual degradation to oligopeptides and amino acids). The basic architecture of plant ATP-dependent proteases is very similar to the architecture of bacterial enzymes, such as those in Escherichia coli, but plastid enzymes apparently have extraordinary numbers of isomers. Recent molecular genetic characterization in Arabidopsis has identified differential roles of these isomers. This review covers what is currently known about the types and function of plastid proteases together with our new observations.
INTRODUCTION

Proteins in living organisms can exist in various forms, either as monomeric or multimeric, soluble or membrane-bound, activated by processing or modification, properly assembled, or as a complex. Each protein thus has its own lifetime, but generally does not exist beyond the lifetime of the organism. This means that most of the proteins are destined to turnover and that protein quality control is physiologically essential for sustaining life (30, 127). The mechanism involved requires protein degradation and regeneration by proteases and chaperone activities, respectively.

Plastids, which are derived from endosymbiosis of photosynthetic bacteria, are plant organelles whose morphology dynamically changes in response to developmental status and the abiotic environment. Examples of plastids are chloroplasts in leaves under light, etioplasts in leaves in darkness, amyloplasts in roots, and chromoplasts in fruits. Accumulating evidence indicates that proteases play an essential role in the transition of one plastid type into others (1, 3). Moreover, the photosynthetic apparatus in chloroplasts undergoes constant photooxidative damage under light, and damaged proteins must be removed by proteases involved in the repair cycle (10, 11, 68, 78; see also 7).

The function of proteases is often vital and versatile. Some proteases act as a chaperone whose activity is inducible under specific conditions. Also, protease activity itself can be regulated in a specific manner such as by endopeptidic processing. Thus, regulated proteolysis can be regarded as fine tuning at the last step of gene expression (30, 125). The ubiquitin-dependent degradation pathway through 26S proteasomes provides a regulatory circuit with many developmental phenomena in plants (72, 113).

In contrast, plastids do not possess 26S proteasomes but instead carry prokaryote-related ATP-dependent proteolytic machinery. While there have been many biochemical attempts to identify the factors involved in plastid protein degradation, only a few have been successful. In the past decade, however, the sequencing of many plant genomes has revealed genes encoding prokaryotic-like proteases that have been well characterized in Escherichia coli, Synechocystis, and other model microorganisms. In this article, I focus on protease families recently identified in plastids of Arabidopsis and other higher plants. I emphasize the prokaryotic ATP-dependent proteases that have been characterized in genomics and molecular genetic approaches. Readers also should refer to recent reviews for chloroplasts (1, 3) and for cyanobacteria (90, 116). Other reviews (4, 20, 41, 94, 114) are also recommended for specific proteases.

TYPES AND FUNCTIONS OF PLASTID PROTEASES: A GENERAL OVERVIEW

How many types of proteases are found in plastids? Although a limited number of proteases have been identified in biochemical
methods, novel protease homologs that are targeted to plastids can be predicted based on the complete genome information from model plant species. Using four programs to predict plastid targeting, Richly & Leister (91) estimated that in Arabidopsis, approximately 2200 proteins constitute chloroplasts along with those encoded in the chloroplast genome. The functions of approximately 5% of these proteins are thought to be related to protein fate. As described below and in Figure 1, at least 11 different types of

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Figure 1
Schematic view of types and functions of proteases in plastids. The upper part shows an electron microscopic image of Arabidopsis chloroplasts. Biogenesis of chloroplasts from proplastids through membrane fission requires import of cytosolic proteins as a precursor and concomitant processing. Once chloroplasts are formed, quality control of proteins is accomplished mainly by ATP-dependent proteases and is crucial for its maintenance. Processive degradation is needed for the transition between plastid types, particularly at sink-source transition and senescence. The bottom part is a schematic representation of proteases present in chloroplasts.
peptidase or protease families encoded in more than 50 genes are found, representing roughly 2.3% of the constituent proteins. The different types of proteases include serine, metallo-, and possibly cysteine, and aspartic proteases, and some of their reaction requires ATP. Most of the peptidases identified in cyanobacteria have paralogs in Arabidopsis, suggesting that the majority of the protease components are of prokaryotic origin (65).

Proteases (or peptidases) in plastids have two types of function. The first function is a positive action of proteases mainly by endopeptidic activities. Some processing peptidases that have been well characterized are stromal and thylakoidal processing peptidases and their related peptidases (94). Plastid-targeted proteins encoded in the nuclear genome are usually synthesized in the cytosol as a precursor with an N-terminal extension (transit peptide). Precursor proteins imported into the stroma undergo maturation by a metallopeptidase termed stroma processing peptidases (SPPs) to remove the transit peptide (71, 92, 93, 134). Further degradation of transit peptides in the stroma involves another type of zinc metalloproteases (termed Zn-MP/PreP) (14, 71). Proteins imported into the thylakoid lumen undergo a similar processing by a peptidase in the thylakoid membrane named thylakoidal processing peptidase (TPP) (16, 106). Another type of the processing occurs in the maturation of D1 protein, one of the two reaction center proteins in Photosystem II (PSII) (7, 79). The processing event in which the C-terminal part of D1 is cleaved off by CtpA (C-terminal processing peptidase) appears to be involved in the proper assembly of the PSII reaction center (42, 95). CtpA is a serine protease with a catalytic dyad (57).

The second function of the plastid proteases is the processive (gradual) degradation of unnecessary proteins to free amino acids. This type of degradation machinery is required for removal of unnecessary proteins that are generated by expression in excess, by error in synthesis and assembly, or by heat denature or photo-oxidative damage. Proteins that have fulfilled their roles may also need to be degraded properly. Plants have developed elaborate machinery for quality control of photosynthetic proteins (particularly the reaction center of PSII), because the chloroplast is where a large number of reactive oxygen species are generated and where the repair of the damaged proteins through degradation and reassembly is crucial for photoprotection (10, 11, 78). Some studies have suggested the plastidic proteases involved in this process are nucleotide-dependent (33, 62, 117). A similar thylakoid-attached proteolytic activity is reported to be involved in degrading the abundant Lhcb proteins in the light harvesting complex of PSII (LHClII) in vitro (25, 28, 129). Degradation of Lhcb and its related proteins is needed to adjust the antenna capacity to light acclimation and also in senescence (5, 60, 135). As implicated in these works, most energy-dependent proteases identified in the past decade possess an ATPase domain that forms a ring structure (see below) and that possibly acts as an unfoldase. I therefore focus on these prokaryotic ATP-dependent proteases in the following sections.

### ATP-DEPENDENT PROTEASES

#### Common Features

Table 1 lists three ATP-dependent proteases experimentally shown to reside in plastids. These proteases, Clp, FtsH, and Lon, share a conserved ATP-binding motif but possess a different catalytic domain for proteolysis. They are all prokaryotic and were originally identified and characterized in E. coli (29). Eukaryotic organisms have homologs of these proteases in plastids and mitochondria. The importance of ATP-dependent proteases in plastids is shown by the fact that chloroplast genomes of not only alga but also higher plants contain a gene potentially encoding ClpP, a protease subunit of Clp (66). Subsequently, ClpP was also discovered in the...
### Table 1  Subunits and isomers of ATP-dependent proteases in plastids of *Arabidopsis*

<table>
<thead>
<tr>
<th>Subunit or isomer</th>
<th>AGI number</th>
<th>Function</th>
<th>Length (amino acid)a</th>
<th>Expression in high lightb</th>
<th>Mutant phenotype</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td>Clp Protease core</td>
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<tr>
<td>ClpP1 (Chloroplast gene)</td>
<td>Atcg00670</td>
<td>Serine protease</td>
<td>196</td>
<td>N.D.</td>
<td>Lethal (heteroplasmy in tobacco and <em>Chlamydomonas</em>) (40, 54, 108)</td>
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<tr>
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<td>At1g66670</td>
<td>Serine protease</td>
<td>309</td>
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<td>ClpP4</td>
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<td>ClpP6</td>
<td>At1g17150</td>
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**Notes:**
- The numbers of amino acids were based on gene annotation and contain transit peptide sequences.
- Expression at the transcript level is based on oligonucleotide microarray analysis by Sinvany-Villalobo et al. (111). −, no induction; +/−, moderate induction; +++, very strong induction. N.D., not determined.
- Personal communication by K. van Wijk.
- Personal communication by F. Myouga and K. Shinozaki.
- Personal communication by H. Janska.

The overall architecture of the Clp complex resembles that of the multimeric 26S proteasome in the cytosol, suggesting that these ATP-dependent proteases are evolutionarily related and are the main protein degradation machineries in plastids (20, 127). A membrane-bound ATP-dependent protease, FtsH, is also encoded in the chloroplast genome in the red algae *Cyani-dioschyzon merolae* (44).

The conserved ATPase domain in the aforementioned proteases includes the
AAA (ATPases associated with various cellular activities) domain: a conserved domain consisting of ~230 amino acid residues found in all prokaryotic and eukaryotic organisms

SRH: second region of homology

Catalytic triad: a tri-amino acid structure that forms a structurally conserved catalytic domain that is characteristic of serine proteases

Zinc-binding motif: a conserved motif found in zinc-requiring metalloproteases consisting of His-Glu-X-X-His

GFP: green fluorescent protein

so-called AAA domain (9, 55, 85). Proteins containing these domains comprise a large protein family called AAA+ (ATPases associated with various cellular activities plus) (76, 80). Among the AAA+ proteins is a subfamily (called AAA proteins) that contains an additional C-terminal conserved domain [second region of homology (SRH)] (47). FtsH is also called AAA protease. AAA+ proteins are universally found in prokaryotes and eukaryotes with various cellular functions (26). The protease domain of Clp is of the serine type and consists of a catalytic triad. Similarly, Lon is a serine-type protease and a recent structural analysis indicates that the catalytic center is a dyad. FtsH is a metalloprotease and the catalytic center is composed of a conserved zinc-binding motif. All three proteases exist as a protein complex, with the ATPase domain forming a hexameric or heptameric core ring structure. Although the precise action of the energy-dependent process with respect to proteolysis is not fully understood, in Clp the ATPase complex recruits and simultaneously unfolds a substrate protein through the ring, and then delivers it into the proteolytic chamber for degradation (127). Although the basic architecture of each protease established in E. coli and cyanobacteria has been described, here I only deal with plastid homologs.

Some ATP-dependent proteases in plastids (e.g., Clp and Lon) are involved in protein degradation in stroma, and others (e.g., FtsH) are involved in protein degradation in thylakoid membranes. Other proteases like Deg may be also involved in the thylakoidal lumen. The presence of these bacterial proteases was initially demonstrated by immunological analysis (83). In chloroplasts, protein complexes undergo complete degradation when lacking either one of the subunits by mutation (128). Accumulation of precursor proteins imported into chloroplasts is usually undetectable in vivo. These proteases may have roles in degrading precursors, excess subunit proteins, and photooxidatively damaged proteins (63, 82). Recent molecular genetic characterization in Arabidopsis has demonstrated the fundamental roles of ATP-dependent proteases, which I describe in detail below.

**Multiplication of Protease Components**

One important feature of plastid proteases as opposed to proteases in other compartments is that their subunits and isoforms exist as multiple copies (Table 1). Although the genes encoding Clp subunits, FtsH, and Lon exist as single copies in E. coli and most eubacteria, the corresponding genes exist as multiple copies in photosynthetic bacteria, unicellular alga, and higher plants (2, 116). Given that some isoforms of these proteases are also present in mitochondria, one should carefully distinguish which ones indeed function in plastids.

Prediction programs are available, but the actual location needs to be confirmed experimentally. Two in vivo ways to localize different homologs in either plastids or mitochondria have been reported by (a) green fluorescent protein (GFP) transient assay using putative N-terminal transit peptide (101), and (b) proteome analysis and determination of the polypeptide in plastid fraction (86, 87, 111, 130). The finding that the number of the subunits and isoforms is greater in higher plants than in unicellular alga and cyanobacteria implies that the diversification occurred during the evolution of photosynthetic organisms. Because many isomers are found in Clp subunits, FtsH, and Lon in Arabidopsis and other plants, we should clarify the nomenclature for these proteins. Efforts have been made to unify their nomenclature, but it should be updated. In addition to the nomenclature proposed by Adam et al. (2), I followed Clarke et al. (20) for Clp, and Huesgen et al. (41) for Deg (previously termed as DegP).

**Clp PROTEASE**

**Basic Structure**

Clp is a multisubunit enzyme in which the catalytic domain for proteolysis and the
ATPase domain are split in different subunits. In *E. coli*, the protease subunit ClpP forms a heptameric ring with a narrow central pore (126). Two of the rings associate with each other on one side and result in forming a tetradecameric barrel-like structure. This has an internal central proteolytic chamber composed of a catalytic triad conferring serine-protease activity. Another complex of the ATPase subunits, which consists of either ClpA or ClpX (containing two or one AAA domains, respectively), is connected further outside of this proteolytic complex at one or both ends (32, 39). ClpA and ClpX independently form a homo-hexameric ring characteristic of AAA+ proteins, and can act, per se, as a chaperone. In combination with ClpP, ClpA/P, and ClpX/P holo enzymes perform protein degradation, in a way that ClpA or ClpX provide an ATP-dependent unfoldase activity and act as a gateway to recruit the unfolded substrates into the narrow proteolytic chamber of ClpP (127). This unfolding-coupled processive degradation is a common mechanism shared with the eukaryotic 26S proteasome and partially with other ATP-dependent proteases, such as FtsH and Lon.

**Subunits and Gene Family**

In *Arabidopsis*, about two dozen genes encoding subunits of Clp have been found (20, 116): Only ClpP1 is encoded in the chloroplast genome and all other genes are in the nuclear genome (*Table 1*). Earlier studies using immunological detection demonstrated the in vivo presence of a complex formed by a protease subunit and a chaperone subunit showing ATP-dependent proteolytic activity (81, 115). Peltier et al. (86) recently identified the component of the Clp protease core complex (325–350 kDa) that resides in stroma and showed that it contains five ClpP isomers (ClpP1, P3, P4, P5, P6), four proteolytically inactive subunits ClpR1 to ClpR4, and two plant-specific subunits ClpS1 and S2. Characterization of the complex in different tissues suggested that the overall composition appears to be similar in different plastid types. The Clp protease complex in plastids, likely forming an authentic tetradecamer, thus is hetero-oligomeric rather than homo-oligomeric. In addition, ClpS1 and S2 peripherally attach to the twin-barrel structure of the core, likely acting as a regulator of the interaction with the chaperone complex. Although the function of ClpR remains unknown, a three-dimensional model of the hetero complex implies that the inclusion of ClpR in the plastid complex may control access of the substrate to the catalytic chamber.

Compared with the protease complex, the chaperone (ATPase) complex is less understood. Nevertheless, plastids contain homologs related to *E. coli* ClpA, termed ClpB3, ClpC1, ClpC2, and ClpD, all of which have two AAA domains and belong to the Clp/HSP100 family, but do not contain homologs related to *E. coli* ClpX (20, 86, 116). Instead, ClpX1-3 appears to reside in
mitochondria exclusively (35), although the prediction programs suggest the possibility that all three are localized in plastids. ClpD was originally identified as a gene highly responsive to drought stress and senescence (75). ClpC (also termed Hsp93) was independently shown to be associated with the translocon Tic of the inner envelope (77). In contrast to plastids, the Clp complex structure in mitochondria seems rather homogeneous and resembles the *E. coli* ClpX/P. The protease core in mitochondria of *Arabidopsis* contains only ClpP and the ATPase complex consists only of ClpX (X1, X2, X3) (86). Homogeneity of the mitochondrial Clp also illustrates that the plastid Clp has exceptional subunit compositions and an extraordinary number of isomers, providing an intriguing question of the relationship between Clp diversity and the evolution of photosynthesis.

Expression and Genetic Analysis

ClpP is dispensable in *E. coli*, but its expression is affected by certain stress conditions like heat shock and carbon starvation (29). Similar observations have been reported in *Bacillus subtilis* and other nonphotosynthetic bacteria. In contrast, ClpP in *Synechococcus* is not responsive to heat shock, but rather plays roles in steady-state growth and acclimation to high-light condition (21, 89). In *Arabidopsis*, most of the Clp genes showed increased transcript levels under high light but were less responsive to temperature shifts (111, 133). These results together with the proteome analysis imply that all isomers constituting the protease core complex substantially accumulate in all plastid types, and that regulation of the proteolytic activity of the complex may depend on regulatory subunits.

Reverse genetic approaches have been taken to further decipher the Clp function in plastids, despite the presence of many isomers (Table 1). Inactivation of ClpP1, encoded in the chloroplast genome, was reported to result in heteroplasmacy between the mutated and wild-type genes in tobacco (54, 108) and *Chlamydomonas* (40), demonstrating that ClpP1 is essential for cell viability. A tobacco plant that retained *clpP1* heteroplasmy showed defective development in mesophyll-containing chloroplasts with disrupted inner membrane structures. No information is presently available on the effects of knockout of nuclear-encoded ClpPs. On the other hand, knockdown of ClpP4 by antisense methods caused severely reduced growth with chlorotic leaf tissues (20). This may mean that each isomer is required for the functional protease core, supporting the hetero-oligomeric composition proposed by Peltier et al. (86), but further genetic evidence is needed. As for other subunits, visible phenotypes such as yellow and pale green have been reported on the knockout or knockdown mutants of ClpR1, ClpR2, ClpR4, ClpC1, and ClpB3, whereas no clear phenotypes were observed on those of ClpC2, ClpD, and ClpT (20, 22a, 51b, 112). Interestingly, a mutation at the *ClpC2* locus was recently shown to suppress a variegated phenotype caused by loss of an FtsH (84). Although the mechanism involved in this genetic suppression remains unclear, it demonstrates the interaction of the Clp regulatory components with other protease members.

FtsH PROTEASE

Basic Structure

FtsH (also called HflB) was originally identified in *E. coli* as an essential protein (123) and was shown to degrade various short-lived proteins (6, 38, 124; see 43 for review). In contrast to multisubunit-composed Clp, FtsH has both ATPase and protease domains in one polypeptide and forms a homo-oligomeric complex. The homologs of FtsH have also been studied in detail in yeast mitochondria, in which three homologs (Yta10-12p) are present in inner membranes (55). Based on an X-ray crystallographic analysis, the ATPase domain of *E. coli* FtsH was proposed to constitute a hexameric ring that contains conserved aromatic residues facing the ring structure and...
the ATP-binding pocket is located between the neighboring protomers (53). The AAA domain of FtsH in plastids is almost identical in *E. coli*, implying a similar hexameric structure in plastid FtsHs.

*E. coli* FtsH has two transmembrane domains at the N terminus that anchor it in plasma membrane, and the protease domain at the C terminus faces the cytoplasm (43). FtsH exists as a very large complex by interacting with other membrane proteins such as HflKC (43, 97). In chloroplasts, all FtsH homologs are located in thylakoid membranes and the protease domain faces the stroma (19, 59, 101). FtsH also exists in plant mitochondria (51). However, yeast has two kinds of AAA proteins, one with a single transmembrane domain (Yta11p) and the others with two (Yta10p and Yta12p). These two proteins have different topologies with respect to the mitochondrial inner membrane: the protease domain faces either the intermembrane space (i-AAA) or the matrix (m-AAA) (9, 55). The two m-AAA proteins form a large hetero complex with other components called prohibitins (8, 120).

### Gene Family

Multiplication of the genes coding for FtsH is much greater in photosynthetic organisms than in nonphotosynthetic bacteria and mitochondria of eukaryotes (64). Four isomers of FtsH have been identified in *Synechocystis* sp. PCC 6803. Inactivation of two of these isomers is lethal and so these isomers appear to be essential (64). Loss of one of the remaining two isomers, slr0228, leads to a dramatic sensitivity to high-light exposure and deficiency in the photosynthetic apparatus in thylakoid membranes, particularly in PSII (110, 51a). FtsH seems to be the main protease factor involved in the repair cycle of PSII, particularly the repair of D1 protein (12, 78). *Arabidopsis* has twelve *FtsH* genes (*FtsH1–12*). In addition, four other genes apparently encode proteins homologous to FtsH, but these are not regarded as FtsH because they lack a conserved zinc-binding motif, which presumably renders them inactive for proteolysis (116). Nine of the 12 FtsHs reside in chloroplasts (FtsH1, 2, 5, 6, 7, 8, 9, 11, 12), and the remaining three reside in mitochondria (FtsH3, 4, 10), based on our GFP transient assays (101). However, FtsH11, which contains only one transmembrane domain, is highly homologous to yeast mitochondrial Yta11, and was recently suggested to be located in both chloroplasts and mitochondria (37, 124a). For all the FtsHs immunologically detected in thylakoid membranes, the proteolytic domain was shown to face the stroma (59).

The importance of FtsH in chloroplasts was originally revealed by immunological detection of FtsH homologs in spinach chloroplasts, followed by isolation of the corresponding cDNA in *Arabidopsis* (59). In an in vitro import assay where a precursor of Rieske Fe-S protein was used with isolated chloroplasts, the precursors that remained in the stroma were promptly degraded, whereas the responsible protease appeared to have characteristics similar to FtsH and its degradation was inhibited by an FtsH antibody (82). As in *Synechocystis*, FtsH in chloroplasts apparently plays an important role in the degradation of photo-oxidatively damaged D1 and other proteins in PSII. Lindahl et al. (58) reported that in isolated spinach thylakoid membranes exposed to high light, D1 is degraded in two distinct steps (during generation of the intermediate products and during subsequent degradation). They found that a recombinant FtsH1 fusion protein expressed by *E. coli* can act on the lateral step. Tobacco DS9 is a homolog of FtsH and is the most similar to *Arabidopsis* FtsH2 (105). It was identified as a protein that was dramatically reduced at the transcript in response to the N gene-mediated hypersensitive response to tobacco mosaic virus. These observations indicate that FtsH in chloroplasts has multiple cellular functions, as is the case in *E. coli* and other microorganisms.
Arabidopsis leaf-variegated mutants called yellow variegated (var). (Top panel) Photographs of wild-type Columbia and var1-1, var2-6, and a suppressor mutant of var2 (sup52). Leaf variegation in var1 and var2 is a result of loss of FtsH5 and FtsH2, respectively. Degree of leaf variegation is generally higher in young leaves than in lateral leaves, and also higher in var2 than in var1. (Bottom panel) Ultrastructures of plastids in var2 at the different developmental stages. A proplastid in shoot meristem (a) appears normal, whereas differentiation into chloroplasts in leaf primordia results in aberrant plastid morphologies with vacuolated inner structures (b and c). In mesophylls of variegated leaves, normal-appearing chloroplasts accumulate in green tissues (d), whereas plastids with collapsed inner membrane structures are detected in white tissues (e). Bar: 0.3 μm in a, b, and c; 1 μm in d and e.

Expression and Genetic Analysis

FtsHs in chloroplasts are one of the best-characterized proteases in terms of molecular genetics. This is because a mutation at genes encoding some FtsHs shows a typical leaf-variegated phenotype that has long been known in Arabidopsis (98). The mutants are called yellow variegated1 (var1) and var2 (shown in Figure 2), and the corresponding loci in the wild type have been shown to encode FtsH5 and FtsH2, respectively, by Steve Rodermel’s group and my group (19, 100, 122). Knockout mutations of the other chloroplastic FtsHs such as FtsH1, 6, and 8 did not result in any visible phenotypes (101). Two important observations here are that (a) loss of one FtsH does not cause
demonstrated that the FtsH complex of Arabidopsis is FtsH2 relative amount estimated at the protein level the complex remained unclear, although the lack of either one resulted in the concomitant decrease of the other (101). A concomitant decrease of FtsH5 in var2 was also observed in a proteomics analysis (130). Regulation likely occurs at the post-translational level. FtsH2 and FtsH5 were found to form a hetero complex of ∼400 kDa.

Proteomic analysis of isolated thylakoids in Arabidopsis demonstrated that the FtsH complex had the highly related homologs FtsH1 and FtsH8, in addition to FtsH2 and FtsH5, which shows that these are the four major FtsHs constituting the FtsH complex (27, 111, 130). Stoichiometry of each isomer in the complex remained unclear, although the relative amount estimated at the protein level is FtsH2-FtsH5-FtsH1-FtsH8 (111). The variegated phenotype observed in var1 (weak) and var2 (severe) seems to correlate well with this observation (Figure 2). Phylogenetic analysis clearly indicates that FtsH homologs can be grouped into at least several clusters. Besides the mitochondrial isomers, two clades can be drawn from this, one with FtsH1 and FtsH5 and the other with FtsH2 and FtsH8. Interestingly, the existence of this pair appears well conserved in unicellular alga (69, 70) as well as in higher plants (Arabidopsis and rice, see 131). Leaf variegation in var2(fsh2) was rescued by overexpressing FshH8 cDNA (130, 131). Similarly, leaf variegation in var1(fsh5) was rescued by overexpressing FshH1 cDNA but not FshH2 cDNA (131), suggesting that FtsH proteins in each clade are functionally redundant. Detailed characterization of the knockout mutants indicated that the appearance of white tissues were more intense in ftsb1/fts b5 and ftsb2/fts b8 double mutants than in ftsb2/fts b5 double mutants (132a).

Yu et al. (131) also demonstrated that antisense repression of FtsH1 in var1(fsh5) background results in albino-like phenotypes. Together, these observations provide an interesting possibility that at least one isomer in each pair may be required for proper function.

Most of the variegated mutants isolated so far turned out to be alleles of var2 (99). A fundamental question is why a distinct variegated phenotype can be observed only with the simultaneous loss of FtsH2 and FtsH5. The appearance of variegated sectors is higher at the juvenile leaf stage than at the later developmental stages. Expression of other chloroplastic proteins such as other FtsHs and ClpC seemed to increase in the late developmental stages (132). Leaf variegation can be suppressed by trans-action of a second-site mutation. These suppressors have been isolated and one has been assigned to the ClpC locus (84). We also recently cloned one of the suppressors and assigned it to be the locus of the gene encoding a plastidic translation initiation factor (unpublished results; see Figure 2). Although these data still do not explain the mechanism leading to leaf variegation, they imply that the function of FtsH in chloroplasts is associated with various photosynthetic activities.

Expression of FtsH isomers at the transcription level indicates that some chloroplastic FtsHs, such as FtsH8, are highly inducible by high light (111). However, the overall FtsH protein levels do not substantially respond to stress conditions. A notable role of FtsH in chloroplasts is its involvement in the PSII repair cycle. In Synechocystis, FtsH has been copurified with PSII complexes (48, 110). Chlorophyll fluorescence induction analysis in green sectors of var1 and var2 showed that these mutants greatly reduce PSII activity when irradiated under high light (12, 100, 132). In fact, high-light-dependent degradation of D1 did occur in the wild-type leaves but not in var2-2 leaves (12, 19). These observations, together with the data provided in cyanobacteria, demonstrate that FtsH is a
Figure 3
GFP-transient assay using suspension-cultured tobacco cells with a Lon4-GFP fusion gene. (Top panel) A protoplast of chlorophyll SC cells was transformed with a GFP gene fused to a putative transit peptide from Arabidopsis Lon4. GFP signals were colocalized with chlorophyll autofluorescence, suggesting that Lon4 targets chloroplasts. In addition, GFP signals were detected as granule bodies that likely corresponded to mitochondria. (Bottom panel) In fact, Lon4-GFP expressed in non-chlorophyllic SL cells colocalized with the signal from a mitochondria-specific probe, MitoTracker.

Lon PROTEASE
Lon was the first ATP-dependent protease found in E. coli. Deficiency of Lon results in accumulation of abnormal proteins and elevated sensitivity to DNA damage (31). Like FtsH, it contains the AAA and protease domains in one polypeptide but no transmembrane domain (96). Characteristic to Lon is its affinity to DNA, although the domain required for DNA binding is unknown (121). A ring-like structure with heptamer has been suggested in yeast mitochondria (119). Lon is therefore considered to form a complex typical of AAA proteins. A crystal structure of E. coli Lon was also determined recently and shown to form a hexameric ring with a catalytic Serine-Lysine dyad (15).

Homologs of Lon have been identified in higher plants (13, 102). In Arabidopsis, four genes have been detected as potentially encoding Lon based on a homology search (Lon1–4) (46). One of the four Lon proteins, Lon1, is specifically involved in the degradation of mitochondrial proteins during tapetum formation in anthers (102). Lon2 has a consensus-targeting signal to peroxisomes at the C terminus (called PTS1) and is phylogenetically associated with animal paralogs that were recently shown to reside in peroxisomes. A GFP assay using Lon2 targeted the protein into peroxisomes (A. Kato, unpublished results). A gene encoding Lon is not found in the genome sequence of Synechocystis sp. PCC 6803. Thus, whether Lon is present in chloroplasts remains controversial. However, a transient assay in my laboratory using tobacco cells with an N-terminal sequence from Lon4 (encoded in At3g05790) fused to GFP showed that Lon4 can be targeted to both chloroplasts and mitochondria (Figure 3). This result, together with immunological detection of Lon homologs in the stroma, indicates that Lon4 is most likely present in plastids and plays roles in protein degradation.

OTHER PROTEASES
E. coli has another type of ATP-dependent protease, HslUV, but no homologs of HslUV have been detected in photosynthetic organisms. Besides Clp, FtsH, and Lon,
ATP-independent protease of the Deg family play roles in protein degradation in plastids (41). DegP is a serine protease that forms a homotrimeric oligomer in *E. coli* and human (22). Two of the trimers further dimerize to form a hexamer (52). *E. coli* has three Deg proteases, Deg P, Q, S, each of which has one or two characteristic PDZ domains toward the C terminus (one in DegS and two in DegP and DegQ) that are necessary for protease-protease interactions and that possibly regulate recognition of substrate proteins. DegP also has a chaperone activity, and the switch between protease and chaperone activities can be accomplished by a temperature shift (24, 118). Similar to AAA proteases, Deg homologs are organized as a protein family in photosynthetic organisms (50, 109), with 16 homologs found in *Arabidopsis* based on the genome information (41, 116). Four of the *Arabidopsis* Deg homologs (Deg1, 2, 5, 8) are located in chloroplasts (17, 36), where they are peripherally attached to the thylakoid membrane. Deg1, 5, and 8 have been located on the luminal side and Deg2 has been located on the stromal side (36, 45, 104). No reports have been made so far on the effects of homozygous knockout mutations in any Deg genes in *Arabidopsis*. Expression of Deg proteases seems to be increased by abiotic stress such as salts, light, and temperature. Deg1 and Deg2 have been studied in detail in *Arabidopsis* (17, 36). In particular, DegP2 has been proposed to play a role in PSII repair through degrading photodamaged D1 in combination with FtsH, although genetic data confirming this role is lacking. Deg appears to play an indirect role in D1 degradation in *Synechocystis*. However, a mutant lacking all Deg proteases shows a slight sensitivity to high light (109), which raises the possibility that the PSII repair system in *Synechocystis* is different from that in *Arabidopsis* (78).

Other proteases have been identified in plastids, but their roles in protein degradation are poorly understood. EGY1 is an ATP-independent metalloprotease that is involved in chloroplast development (18). EGY1 has eight putative transmembrane domains presumably located in thylakoid membranes, and more interestingly, its catalytic center, represented by a zinc-binding motif, seems to be embedded inside the membrane. The function of EGY1 may be similar or equivalent to that of Rhomboid protease in yeast mitochondria (67), although homologs of Rhomboid other than EGY1 and EGY2 have been detected in *Arabidopsis*. Both cyanobacteria and chloroplasts possess homologs of bacterial SppA tightly associated with thylakoid membranes (56, 88, 114). SppA is an ATP-independent serine protease whose expression appears to be upregulated by light. CND41 is a tobacco protein identified as comprising nucleoid (it has an affinity for DNA and is located in the stroma) in suspension-cultured cells (74). It has an aspartic protease activity and is induced by senescence (49, 73). *Arabidopsis* has two homologs whose functions are currently unclear. A stromal glutamyl endopeptidase (GEP) that can cleave in vitro a short peptide corresponding to the N-terminal part of Lhcb1 was recently found in pea (25). A protein homolog of GEP is present in *Arabidopsis* and appears to have a potential triad for serine proteases.

**FUTURE PERSPECTIVES**

As described in this review, the recent availability of genome information in model plant species has made it possible to determine the overall architecture of the proteolytic machineries in plastids, without knowing the substrates and biochemical properties the proteases involved. The identified proteases substantially accumulate in plastids at the basal level, meaning that their proteolytic activities must be strictly regulated. A combination of genetic and biochemical approaches, such as the use of T-DNA insertion mutants and suppressors, should greatly elucidate the regulatory mechanism. The phenotypes of these mutants (especially those without obvious phenotypes) need to be observed under different growth conditions and at different
development stages. Some questions that need to be answered are (a) does the lack of any one or multiple proteases affect plant growth under certain temperature/light conditions, and (b) can a protease act as a regulator of specific plastid types in different tissues? In addition, a comparison of the sequences and functions of the protease components in unicellular and multicellular organisms other than Arabidopsis will show how these machineries have evolved or have been conserved.

Many questions need to be answered. Although the basic structure of plastidic complexes is known, the proteolytic action of ATP-dependent proteases is poorly understood, mainly due to their low proteolytic activity in vitro. Determining the substrates of the proteases, and whether they are specific substrates is a challenging but important task. For example, FtsH is known to degrade photodamaged D1, but how it recognizes photodamaged or denatured substrates is unclear. Apparently, turnover of abundant proteins in chloroplasts such as Rubisco and Lhcb must be taken into consideration. If FtsH or Clp specifically degrades one of the sigma transcription factors in plastids, then these proteins may regulate chloroplast development. To fully understand the regulatory mechanism, characterization of protein profiles in the knockout mutants and in the different plastid types will help to identify the proteins involved in the key processes.

**SUMMARY POINTS**

1. Plastids contain more than 11 types of proteases. In Arabidopsis, these proteases are encoded by more than 50 genes.
2. Plastids contain three prokaryotic ATP-dependent proteases, Clp, FtsH, and Lon, as major enzymes for processive degradation.
3. Many isoforms of Clp subunits are present in plastids and some are plant-specific. The Clp protease core complex is heteromeric and each isomer seems to be required for plant viability.
4. Twelve FtsH isoforms are present in Arabidopsis plastids and form a hetero/homo complex. FtsH is involved in the repair cycle of Photosystem II.
5. Four FtsH isoforms (FtsH1, 2, 5, 8) are the major ones, and the presence of either FtsH1 or FtsH5 as well as either FtsH2 or FtsH8 is necessary for thylakoid development. Loss of FtsH2 and FtsH5 leads to a leaf-variegated phenotype.

**FUTURE ISSUES TO BE RESOLVED**

1. Multiple mutants for protease subunits or isomers need to be characterized, particularly under different environmental conditions. Substrates for each ATP-dependent protease and the mechanism of substrate recognition should be determined.
2. Differential functions of each isoform, especially those specific to plastids (such as ClpR and ClpS), should be examined.
3. Factors affecting proteolytic activity and/or associated with protease complexes need to be identified.
NOTE ADDED IN PROOF

Involvement of FtsH6 in the degradation of LHCII during high-light acclimation and senescence was recently reported by Želisko et al.

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LITERATURE CITED


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The first report on the identification of an FtsH homolog in chloroplasts.


This paper describes the cloning of the VAR1 locus encoding FtsH5, whose mutation results in leaf variegation.

This paper describes the presence of nine FtsH homologs in chloroplasts by GFP assay and describes the formation of the homo/hetero FtsH complexes.


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