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# Proteolysis in plants: mechanisms and functions

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## Contents



## Abstract

Proteolysis is essential for many aspects of plant physiology and development. It is responsible for cellular housekeeping and the stress response by removing abnormal/misfolded proteins, for supplying amino acids needed to make new proteins, for assisting in the maturation of zymogens and peptide hormones by limited cleavages, for controlling metabolism, homeosis, and development by reducing the abundance of key enzymes and regulatory proteins, and for the programmed cell death of specific plant organs or cells. It also has potential biotechnological ramifications in attempts to improve crop plants by modifying protein levels. Accumulating evidence indicates that protein degradation in plants is a complex process involving a multitude of proteolytic pathways with each cellular compartment likely to have one or more. Many of these have homologous pathways in bacteria and animals. Examples include the chloroplast ClpAP protease, vacuolar cathepsins, the KEX2-like proteases of the secretory system, and the ubiquitin/26S proteasome system in the nucleus and cytoplasm. The ubiquitin-dependent pathway requires that proteins targeted for degradation become conjugated with chains of multiple ubiquitins; these chains then serve as recognition signals for selective degradation by the 26S proteasome, a 1.5 MDa multisubunit protease complex. The ubiquitin pathway is particularly important for developmental regulation by selectively removing various cell-cycle effectors, transcription factors, and cell receptors such as phytochrome A. From insights into this and other proteolytic pathways, the use of phosphorylation/dephosphorylation and/or the addition of amino acid tags to selectively mark proteins for degradation have become recurring themes.

## Introduction

The ultimate post-transcriptional control of gene expression involves the proteolytic breakdown of the encoded protein back to its constituent amino acids. Here, protein degradation not only represents an important recycling system for amino acids but also represents the final step in what can be a complex cascade of regulatory events controlling gene function [for reviews see 25, 34, 95, 207]. In the past decade, it has become increasingly obvious that the ability of cells to switch from one developmental state to another or to adapt to new environmental conditions often requires the rapid dismantlement of existing regulatory networks, a process frequently dependent on proteolysis. Examples range from the control of metabolism and cell specification to the progression of the cell cycle and the initiation of various signal transduction pathways. Moreover, both the speed (proteins can have half-lives <5 min) and irreversibility of proteolysis provide advantages to cellular regulation not offered by other mechanisms. With respect to the various post-translational regulatory processes, it should be emphasized that none are more influential or pervasive than protein breakdown in determining the final concentration of active proteins.

The purpose of this chapter is to describe our present understanding of how plant proteins are degraded and to illustrate the various ways that proteolysis can be used as a regulatory mechanism. This review will not focus solely on plants but will also include important paradigms from animal and bacterial systems that have relevance to plant protein breakdown [34, 75, 95, 143]. As will be seen, proteolysis is an intricate process, involving a multitude of pathways to select and catabolize target molecules. It can range from single cleavages that activate (or inactivate) proteins to the total digestion of the polypeptide. From the initial analysis of the few proteolytic pathways identified in plants, it is clear that the level of complexity required to degrade proteins may eventually rival that required to initially synthesize them [25, 207]. For example, we estimate that the ubiquitin-dependent proteolytic pathway alone may involve over 100 genes in *Arabidopsis thaliana* (or 0.5% of the coding region); more than 45 of which have been identified to date [207, R.D. Vierstra, unpublished]. Given that over 10000 proteins can exist simultaneously in any given plant cell, it is also not surprising that cells have evolved highly sophisticated mechanisms for selective target recognition, thus avoiding the indiscriminate breakdown of other proteins.

## Mechanisms for degrading proteins

### *General features*

At first glance, protein degradation would appear to simply involve a protease (or set of proteases) digesting a protein. However, several general observations argued early on that the process cannot be that simple [44, 77, 103]. First, most *in vivo* proteolysis requires energy. Because peptide bond hydrolysis is an exergonic reaction, and because most purified proteases are energy-independent, this requirement presupposed that energy-dependent steps must exist and that they may control proteolysis. Second, protein degradation is fast, so rapid in fact that detecting partial breakdown products is often difficult. This rapidity implies that once the proteolytic machinery finds a suitable target, it uses multiple protease activities to completely digest the target before another target is chosen. The efficient removal of partial cleavage fragments may be physiologically essential as these peptides could interfere with a multitude of protein/protein interactions should they accumulate. However, the failure to detect partial breakdown products also represents a major technical barrier in understanding how proteins catabolized and which proteases are responsible [207].

A third feature is that although most proteases are specific to certain amino acids sequences (e.g. trypsin cleaves after Arg and Lys residues) and/or sites within the protein (i.e. internal peptide bonds [endopeptidases] or terminal peptide bonds [exopeptidases including Nterminal aminopeptidase or C-terminus carboxypeptidases]), they are not typically restricted to specific proteins [12]. In fact, this lack of specificity has created an unusual nomenclature for proteases. Unlike other enzymes which are generally classified according to their substrates or products, proteases are typically classified based on the essential elements within their active sites. Examples include cysteine-, serine-, aspartic acid- and metalloproteases which require the aforementioned components in their cleavage reactions [12]. Because of this broad specificity, most proteases must be regulated or compartmentalized to avoid random breakdown of all intracellular proteins.

The fourth feature is that proteolysis is highly selective. Even in the same cellular milieu, protein

half-lives can range from minutes to weeks [25, 77, 207]. Moreover, the turnover rate of individual proteins can vary dramatically depending on the conformational state and location of the protein, or on the developmental and physiological state of the cell. For example, the half-life of the plant morphogenic photoreceptor, phytochrome A, can vary by ca. 100-fold depending on whether its in the Pr or Pfr forms [208]. This selectivity implies that proteolytic mechanisms exist that individually recognize appropriate targets and that these mechanisms can be regulated. While it was originally thought that the overall physico-chemical properties (e.g. molecular mass, isoelectric point, thermal stability) of proteins govern their half-lives [see 44, 77], it is now clear that the essential determinants are often contained within small, sometimes conserved domains [50, 73, 117, 153, 199, 205, 218]. Several domains that confer a short half-life are functionally transferable to other proteins, thus raising the intriguing possibility that protein half-lives can be rationally re-engineered [73, 117, 199, 205, 218].

Nonetheless, it should be emphasized that not all proteins are continually susceptible to degradation. In fact, the half-life of total protein can be quite long (ca. 4–7 days in non-stressed plants [44, 103, 148, J. Walker and R.D. Vierstra, unpublished]) indicating that only a small percentage of proteins (<10%) undergoes rapid breakdown at any given moment and that most proteins actually turn over very slowly. But, these short-lived proteins are often responsible for rate-limiting steps in metabolic pathways or act as critical regulators [34, 77, 103, 207]. By helping control the levels of these proteins, degradation can have a profound, but energy cost-effective, influence on cell biology.

In the past decade, substantial progress has been made toward our understanding of protein degradation in both prokaryotes and eukaryotes [for reviews see 34, 77, 143, 207]. Evidence has emerged that several distinct pathways exist in plants with each cellular compartment having one or more (Fig. 1). The types of pathways are consistent with the evolutionary origin of each compartment; for example, chloroplasts and mitochondria appear to use pathways similar to those found in prokaryotes whereas the cytoplasm and nucleus have pathways in common with other eukaryotes [25, 207]. Examination of several of these proteolytic pathways has allowed us to answer, at least in a rudimentary way, some of the fundamental questions concerning protein turnover: What is the nature of the energy requirement? Where does proteolysis occur? How does proteolysis occur so rapidly and completely? And, how is proteolysis so exquisitely selective?

*Degradation of protein in the cytoplasm and nucleus*

*Ubiquitin-dependent proteolytic pathway*

Our first insights into how cytoplasmic and nuclear proteins are degraded were made ca. 15 years ago by Hershko and colleagues with the discovery of a major proteolytic pathway involving the small protein, ubiquitin [for reviews see 34, 95, 97]. In this pathway, short-lived proteins are broken down by a multi-subunit protease called the 26S proteasome following their conjugation with multiple molecules of ubiquitin. The pathway was characterized initially using rabbit reticulocytes and subsequently has been shown to exist in a wide range of other eukaryotes, including humans, yeast (*Saccharomyces cerevisiae*), *Drosophila, Caenorhabditis elegans*, *Arabidopsis thaliana*, wheat, and various other plant species [34, 75, 109, 207]. Most, if not all, of the major steps have been elucidated from studies with these organisms and are illustrated in Fig. 3.

Given ubiquitin's central position in the proteolytic pathway, understanding its unusual structure and mode of synthesis has been helpful in determining how the pathway functions mechanistically. As the name implies, ubiquitin is indeed ubiquitous, being present in species from all kingdoms. It is arguably the most conserved protein yet identified; its 76-amino acid sequence is identical among all higher-plant species analyzed to date and differs by only one residue to that in the alga *Chlamydomonas reinhardtii,* by two residues to yeast ubiquitin, and by three residues from the invariant sequence present in animals [26]. Sequence homologues have also been found in an archaebacterium [217] and a eubacterium [54]. Whether the ubiquitin pathway is widely distributed in these prokaryotic kingdoms is unclear; for example, *Escherichia coli* does not appear to contain ubiquitin [143].

X-ray crystallographic structures of plant and animal ubiquitins show that the molecule consists of a compact globular domain with a flexible, protruding C-terminus (Fig. 2). The compact structure is stabilized by extensive hydrogen bonding that accounts for ubiquitin's unusual resistance to acid, base, and heat denaturation and its ability to rapidly refold to its native conformation once unfolded [20, 209]. As will be seen below, the exposed C-terminal Gly-76 participates in a number of essential reactions in the path-

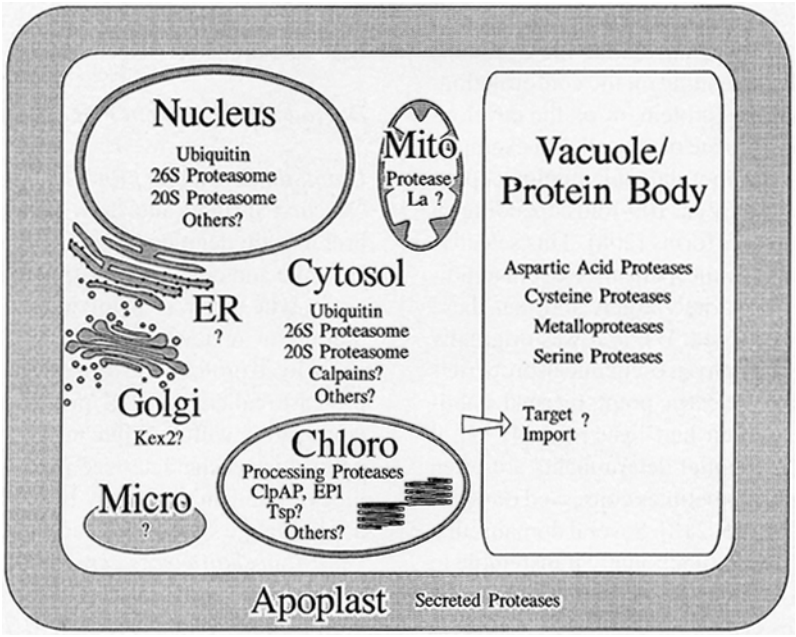


*Figure 1.* Cellular location of various plant proteases and proteolytic pathways in plant cells. Diagrammatic representation of the subcellular compartments within a typical plant cell along with the proteases that have been identified within the compartment. Details of each protease or pathway are described in the text. Question marks denote proteolytic activities or pathways that have been detected but not yet confirmed or proposed but not yet detected in plants. Chloro, chloroplasts; ER, endoplasmic reticulum; Micro, microbodies; Mito, mitochondria.

way. Its substitution or removal [which occurs rapidly in plant extracts by endogenous proteases cleaving at Arg-74 (Fig. 2)] renders the molecule completely inactive [209]. In plants, as in other eukaryotes, ubiquitin is primarily localized in the cytoplasm and nucleus with trace amounts in the vacuole and membrane fractions [15]. It is not present within plastids despite early reports to the contrary [see 15].

Ubiquitin is also unusual among eukaryotic proteins in that it is encoded by complex multi-gene families that synthesize ubiquitin as a natural protein fusion (Fig. 3) [26, 109]. While the reason behind this organization is unclear, its conservation among organisms as diverse as unicellular eukaryotes, angiosperms and mammals, suggests that it serves an important role in ubiquitin synthesis and/or function. In plants, a number of ubiquitin fusion genes have been described; the best characterized family is from *Arabidopsis* where 14 different ubiquitin genes exist (*AtUBQ1–14* [22, 27, 28]). In each case, functional ubiquitin monomers are released from the fusion protein by a unique group of proteases, designated ubiquitin C-terminal hydrolases (or ubiquitin proteases), that specifically cleave the $\alpha$-amino peptide bond that follows the C-terminal Gly-76 of each ubiquitin moiety [26, 109, 97]. Processing is rapid in plants and may occur co-translationally, thus preventing unprocessed ubiquitin fusions from accumulating [63, 100]. The $\alpha$-amino hydrolases are constrained to having ubiquitin sequence at the N-terminal side of the cleavage site, but are unaffected by sequence at the C-terminal side of the cleavage site, provided that proline is not the first residue [205]. The uncommon specificity of these hydrolases has allowed synthetic ubiquitin fusions to be exploited as a novel method to express proteins *in vivo* with N-termini besides methionine [7, 55, 100, 205].

In one type of ubiquitin gene fusion, tandem arrays of ubiquitin coding regions are fused, thus directing the synthesis of a polyubiquitin precursor [26, 109] (Fig. 3). Polyubiquitin genes containing 6, 5, 4 and 3 ubiquitin repeats are present in *Arabidopsis* [22, 28]; two seven-repeat and two six-repeat genes are found in maize [33] and sunflower [17], respectively; two four-repeat and one six-repeat genes exist in flax [2]; and an astonishing 52 ubiquitin-repeat gene was detected in *Trypanosoma* [191]. Although the nucleotide sequences do vary among the ubiquitin coding repeats, each encodes the canonical ubiquitin amino acid sequence. The last repeat of each polyubiquitin gene almost always encodes extensions of one to sever-

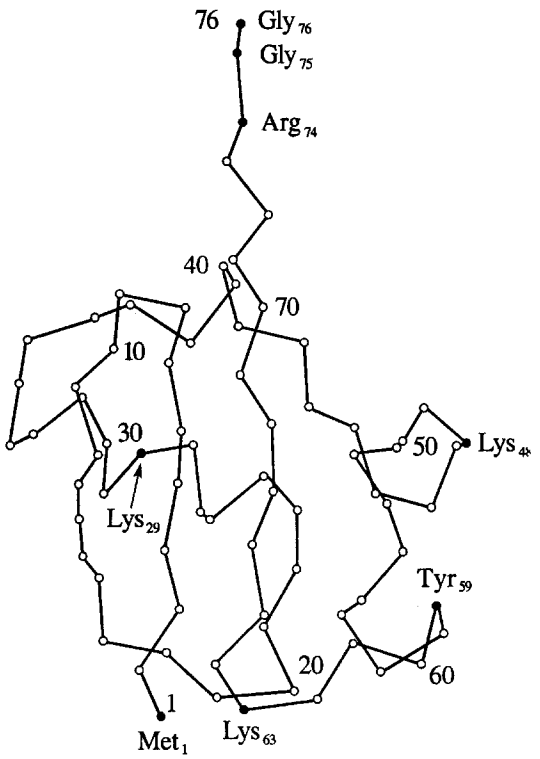


*Figure 2.* Three-dimensional structure of plant ubiquitin as determined by X-ray crystallography [210]. The lysine residues at positions 29, 48, and 63 which can participate in forming multiubiquitin chains are indicated. $Tyr_{59}$ is typically modified with $^{125}I$ to track ubiquitin *in vitro* reactions.

al additional amino acids before the termination codon; these extra residues presumably prevent the ubiquitin pathway from using these polyubiquitin proteins until they are processed into monomers [26, 109].

In another type of gene fusion, single ubiquitin coding regions are appended to the 5′ end of those encoding one of two unrelated ribosomal subunits, thus expressing ubiquitins with long C-terminal extensions [26, 27, 109] (Fig. 2). In plants, these extensions are either 52 or 79–82 amino acids long and are 70–85% identical to counterparts in animals and yeast [27]. Only after removal of the ubiquitin moiety, do these extension polypeptides associate with the ribosome [27, 60]. Because these ribosomal subunits are naturally expressed only as ubiquitin fusions and because they express poorly in an unfused form, it has been speculated that the ubiquitin moiety assists in the translation and/or stability of the subunits prior to their integration into the ribosome [60].

Loci predicted to encode ubiquitin-like proteins have also been found in *Arabidopsis*, yeast, several animals, and Baculoviridae viruses [see 28, 87, 109, 132 and references therein]. Some are organized similarly to the polyubiquitin genes whereas others have a single ubiquitin-coding region with a C-terminal extension. Several of the non-plant versions are expressed and functional. In fact, a human ubiquitin-like protein, whose expression is enhanced by $\gamma$-interferon, can become conjugated to other proteins [132]. A majority of the *Arabidopsis* loci are not transcribed and thus likely represent pseudogenes [28]. However, a cDNA derived from one of these ubiquitin-like loci has been detected in an *Arabidopsis* cDNA library suggesting that some are functional genes [28].

Following synthesis of ubiquitin monomers, the first step in the ubiquitin-dependent proteolytic pathway is the covalent ligation of ubiquitin to proteins destined for breakdown (Fig. 3). This post-translational modification is accomplished by an enzymatic cascade involving ubiquitin-activating enzymes (or E1s), ubiquitin carrier or conjugating enzymes (or E2s), and sometimes ubiquitin-protein ligases (or E3s) [for reviews see 34, 95, 97, 109, 207]. In the first step, an E1 directs the ATP-dependent formation of a high energy thiol-ester intermediate, created by linking the C-terminus Gly-76 of ubiquitin to one of its cysteines [90, 91]. The activated ubiquitin is then transferred from the E1 to a specific cysteine in an E2 via transesterification. Finally, the E2 either ligates the ubiquitin directly to the target protein or transfers the activated ubiquitin to an associated E3 via another transesterification step [170]; the E3, in turn, transfers ubiquitin to the target protein. Studies with a number of eukaryotic species indicate that conjugation is hierarchial [34, 97, 207]. In yeast and *Arabidopsis*, for example, only one or two related E1s execute ubiquitin activation [53, 92, 144], but a multitude of E2s and E3s assist in the transfer of E1-bound ubiquitin to various targets [34, 109, 207]. Ubiquitin is linked to the target protein via an isopeptide bond between the C-terminal Gly-76 of ubiquitin and free lysyl $\epsilon$-amino groups within the target. Structural studies of several ubiquitinated proteins have led to the notion that ubiquitin attachment to the target is often not restricted to contextually specific lysine(s), but in fact can be quite promiscuous [101, 199, K. Lohman and R.D. Vierstra, unpublished].

In a few cases, a single ubiquitin is appended to the target [95, 109]. These monoubiquitinated proteins appear to be metabolically stable, suggesting that adding a single ubiquitin moiety does not commit a protein to degradation but may serve to alter protein

structure or function, possibly in a manner analogous to protein phosphorylation. However in most cases, the conjugation cascade modifies the target protein with multiple ubiquitins [95, 109]. Although this modification could occur by attaching single ubiquitins to different lysine residues within the protein, it most often occurs by attaching one or more chains of ubiquitin monomers. These chains subsequently provide a strong signal for degradation [29]. Multiubiquitin chains consist of ubiquitins linked together through $\epsilon$-amino isopeptide bonds between the C-terminal Gly-76 of one ubiquitin and lysine residues in the adjacent ubiquitin. Lys-48 appear to be the most common residue involved in this intermolecular connection [29, 201]; through interactions among neighboring ubiquitins, these Lys-48-linked chains assemble into compact polymers with 2-fold symmetry [37]. In addition to Lys-48, several studies have implicated Lys-29 and Lys-63 in chain assembly [6, 112, 184]. All three of these lysines are found on the surface of ubiquitin's three-dimensional structure (Fig. 2).

How multiubiquitin chains are generated is unclear but two mechanisms are possible. The chains could be assembled directly on the target by reiterative rounds of ubiquitination or they could be preassembled as free chains and then attached *en masse* to the target in a single step. While current opinion favors the former route, three lines of evidence support the latter as a possible mechanism. First, several E2s have been identified in mammals and plants that can assemble multiubiquitin chains *in vitro* [31, 200]. One family encoded by wheat *TaUBC7* and *Arabidopsis AtUBC7/13/14* genes forms such chains using Lys-48 as the exclusive linkage [200, 203]. Second, free multiubiquitin chains can be detected in a variety of eukaryotes including several plant species and are often the most abundant ubiquitin conjugates present in cell extracts [201]. Third, free chains are as kinetically competent as ubiquitin monomers in ubiquitin conjugation reactions [31, 201]. Collectively, these data imply that the ubiquitin pathway can synthesize free multiubiquitin chains and use them directly in conjugation reactions *in vivo*.

Once a protein is tagged with one or more multiubiquitin chains, it has two possible fates. The ubiquitin moieties can be removed by one of a group of ubiquitin C-terminal hydrolases that specifically cleaves ubiquitins linked via isopeptide bonds [34, 97] (Fig. 3). These $\epsilon$-amino hydrolases are potentially distinct from the $\alpha$-amino hydrolases responsible for processing ubiquitin translational fusions [see above]. While some likely help recycle functional ubiquitins during target degradation by removing the residual peptide fragments from ubiquitin's C-terminus, others can deubiquitinate intact proteins. Recent observations that specific hydrolases are intimately involved in cell division [159] and certain aspects of development (e.g. eye cell fate in *Drosophila* [102]) and that there are 15 or more distinct types of hydrolase proteins in yeast [97] imply that deubiquitination may have important regulatory functions.

Ubiquitin C-terminal hydrolases fall into two broad classes [97]. One class of relatively small proteins (ca. 20 kDa) appear to remove small molecules (e.g. peptides, lysine, and glutathione) from ubiquitin's C-terminus. Surprisingly, one of these comprises up to 5% of the total protein in animal neuronal tissue [215]. Hydrolases in the other class are much larger (50–300 kDa) and cleave ubiquitin from a range of proteins. Some prefer ubiquitins linked via an $\alpha$-amino linkages, $\epsilon$-amino linkages, or can accommodate both. Members that prefer $\epsilon$-amino linkages include yeast DOA4 [159] and mammalian isopeptidase T [88], both of which may function in the disassembly of multiubiquitin chains. In addition to their larger size, the second class is defined by the presence of two conserved motifs, one containing an essential cysteine and the other containing two essential histidines that are necessary for catalysis [102]. Little is know about ubiquitin C-terminal hydrolases in plants. Activities corresponding to both $\alpha$- and $\epsilon$-amino hydrolases have been detected in wheat germ [188]. Recently, several *Arabidopsis* genes have been identified that encode proteins structurally related to the large-size class of hydrolases including the presence of the conserved Cys and His boxes [N. Yan, T. Falbel and R.D. Vierstra, unpublished].

A second fate of ubiquitin conjugates is that they can be degraded by the 26S proteasome, a 1.5-MDa ATP-dependent proteolytic complex specific for such intermediates [95, 161, 193] (Fig. 3). The 26S proteasome degrades the target protein into amino acids and short peptides but releases the ubiquitin moieties in free, functional forms. In this way, ubiquitin serves as a reusable recognition signal for protein breakdown.

*20S and 26S Proteasomes*

The 26S proteasome contains ca. 30 polypeptides that dissociate in the absence of ATP into two subcomplexes of 20S and 19S, both of which are approximately 700 kDa in size [161, 164, 193] (Fig. 4). The 20S particle (known as the 20S proteasome, multicatalytic

protease, or macropain) contains the catalytic core of the protease and is ATP-*in*dependent. It is present in both the nucleus and cytoplasm of animals [95, 193] and plants [25, 171, 193] with related species also found in some archaebacteria [135] and eubacteria [192]. Its distinctive hollow cylinder shape, which can be detected in plant extracts by electron microscopy [156, 171, 193], is created by the assembly of four stacked rings, each of which contains seven polypeptides (Fig. 3).

In the archaebacterium, *Thermoplasma acidophilum*, the subunit composition of the 20S particle is simple, the two outside rings are formed by identical $\alpha$-polypeptides and the two inside rings are formed by identical $\beta$-polypeptides [135] (Fig. 4). Its composition in animals and plants is more heterogeneous, involving as many as 14 different $\alpha$-like and 14 different $\beta$-like polypeptides that range in size from 22 to 35 kDa [156, 193]. Some $\beta$-type subunits are made as larger precursors that require proteolytic removal of an N-terminal extension prior to integration into the complex. In yeast, missense mutations within three different $\beta$-type subunits (PRE1, 2 and 3) leads to a slow growth phenotype, hypersensitivity to stress, and a failure to degrade ubiquitin conjugates, whereas complete disruptions of the corresponding genes are lethal [93, 176, 193]. Genes encoding two $\alpha$-like and one $\beta$-like subunits have been identified in *Arabidopsis* that display greater than 50% amino acid sequence identity to counterparts in yeast and various animals [67, 69, 181]. An *Arabidopsis* line bearing a chromosomal deletion of one of the $\alpha$-like subunits is phenotypically normal suggesting that a multi-gene family exists or that this subunit is non-essential [181]. In spinach, some of the 20S polypeptides may be glycosylated [171].

At least five types of protease activities are associated with the 20S complex, including chymotrypsin-like, trypsin-like, and peptidyl-glutamyl bond hydrolyzing activities [161, 164, 193]. One or more protease activities reside in members of the $\beta$-subunit family which use a novel active-site involving the N-terminal threonine [175]. Several selective chemical inhibitors of the mammalian 20S complex have been discovered that are effective both *in vitro* and *in vivo* [58, 165]. One of these, lactacystin, acts by covalently binding to the active-site threonine in one or more of the $\beta$-like subunits [58]. Whether these inhibitors are also effective in plants is currently under investigation [J. Walker and R.D. Vierstra, unpublished]. While the 20S complex can degrade unfolded proteins completely in the absence of ATP, it has difficulty with native proteins implying that the 19S complex assists as an 'unfoldase' [51]. The cleavage patterns of the purified 20S proteasome is, for the most part, non-specific and typically generates peptide fragments 6–9 residues long [213]. *In vivo*, most of these peptides would then be completely degraded to amino acids by cytosolic peptidases. However, in a special case involving the presentation of foreign antigens in mammals, these peptides are transported to the endoplasmic reticulum (ER) and ultimately to the cell surface where they are presented to the immune system by MHC class I molecules [58, 165]. In mammals, the subunit composition of the rings can be altered by $\gamma$-interferon, suggesting that the catalytic specificity of the 20S proteasome can be modified by developmental or environmental cues [64].

The crystal structure of the 20S proteasome from *Thermoplasma* was recently solved to 0.34 nm [135]. It shows the complex to contain 3 cavities (Fig. 4). The central cavity is created by association of the two equatorial, $\beta$-subunit-containing rings and harbors the active-site threonines [135, 175]. The outside cavities are positioned at the interface between the $\alpha$- and $\beta$-subunit-containing rings and form a narrow channel restricting access to the central lumen. In this way, the site of proteolysis is spatially isolated from the rest of the intracellular milieu in a structure that would allow only unfolded proteins to enter and amino acids and small peptides to exit [76].

The 19S particle binds to one or both ends of the 20S proteasome (Fig. 4). Because it imparts both ATP and ubiquitin dependence to the 26S particle, it is often referred to as the regulatory complex [161, 193] or the 700-kDa proteasome activator (PA700 [45]). Presumably, this complex assists in the recognition of ubiquitinated substrates, unfolds them, and then facilitates entry of the unfolded substrates into the lumen of the 20S proteasome. Electron micrographs of the 19S complex from rat and spinach show an identical V-shaped structure, the interior of which could be the site of protein unfolding [193] (Fig. 4).

The 19S complex contains approximately 15 subunits ranging in size from 35 to 110 kDa [161, 193]. At least five subunits have been identified as members of a newly recognized ATPase family, suggesting that they couple protein unfolding to ATP hydrolysis in a similar manner to chaperonins [45, 161]. Deletions of several of these in yeast arrest cell division [70, 80]. Another subunit, DOA4 in yeast, was recently shown to be a ubiquitin C-terminal hydrolase [159]. This hydrolase appears to regenerate free ubiquitins during the final stages of conjugate digestion as its deletion res-

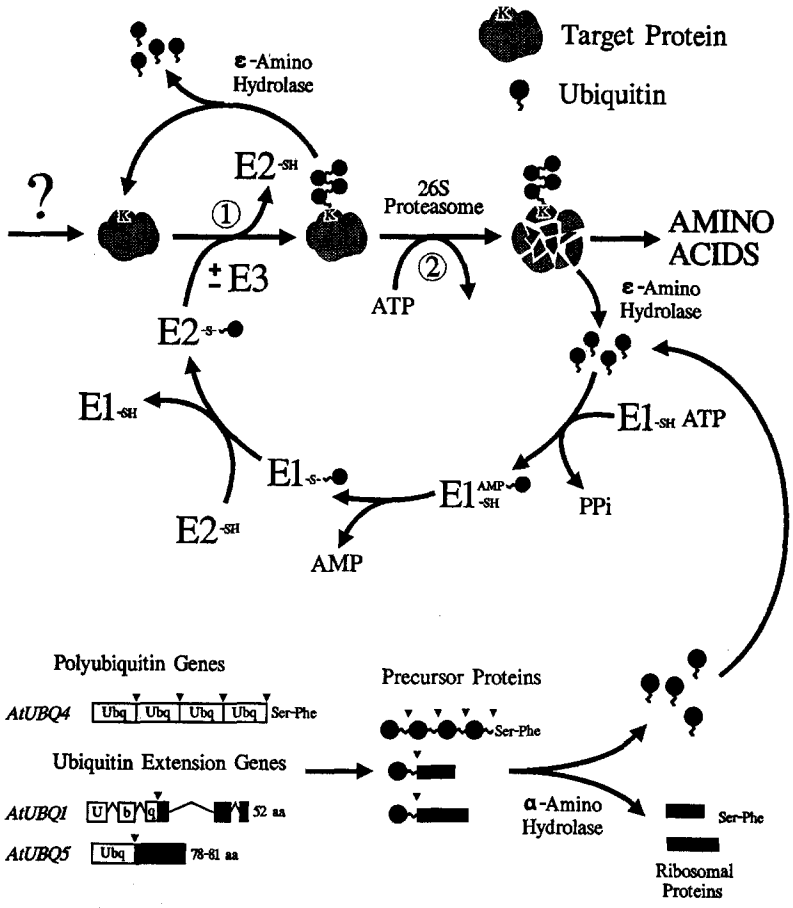


*Figure 3.* Pathway for ubiquitin-dependent proteolysis. The pathway begins with synthesis of ubiquitin fusion proteins, either polyubiquitin or ubiquitin extension, followed by their processing by α-amino ubiquitin C-terminal hydrolases to release ubiquitin monomers. Several of these ubiquitin monomers are then ligated to a protein targeted for degradation using an ATP-dependent reaction sequence involving E1s, E2s, and possibly E3s. The ubiquitinated protein is either disassembled by ε-amino ubiquitin C-terminal hydrolases or degraded to amino acids and peptides by the ATP-dependent 26S proteasome with the concomitant release of free ubiquitin. The ubiquitin genes provided as examples are *AtUBQ1, 4* and *5* isolated from *Arabidopsis thaliana* [22, 27]. K, lysine involved in ubiquitin attachment; Ubq, ubiquitin.

ults in the accumulation of ubiquitin chains linked to small peptides. As expected, the 19S complex also contains a subunit that binds ubiquitin [48, 202]. The gene encoding this 50-kDa polypeptide, designated MBP1 (for *m*ultiubiquitin *b*inding *p*rotein), was first isolated from an *Arabidopsis* cDNA library using free multiubiquitin chains as probes [202]. Sequence analysis subsequently showed MBP1 to be a member of a highly conserved gene family present in a wide variety of other eukaryotes including *Caenorhabditis*, *Drosophila*, man, yeast, rice, and castor beans [202]. The plant 19S particle has been isolated and visualized by electron microscopy [193], but only the MBP1 subunit has been characterized to date (see below).

*Specificity of the ubiquitin pathway*

Within the ubiquitin pathway, two important recognition events occur that determine specificity; the first selects appropriate substrates for ubiquitination, and the second identifies ubiquitin conjugates for breakdown by the 26S proteasome (Fig. 3). The first recognition event encompasses step(s) in the conjugation cascade involving E2s and/or E3s [34, 109, 207].

E2s are a heterogenous family of enzymes, generally ranging in size from 14 to 35 kDa, with distinct substrate specificities and E3 requirements [109, 207]. Structurally, all E2s share a common 150-amino acid core domain that has a pocket containing the essential cysteine required for forming the E2-thiol ester intermediate [36]. Even though there can be substan-

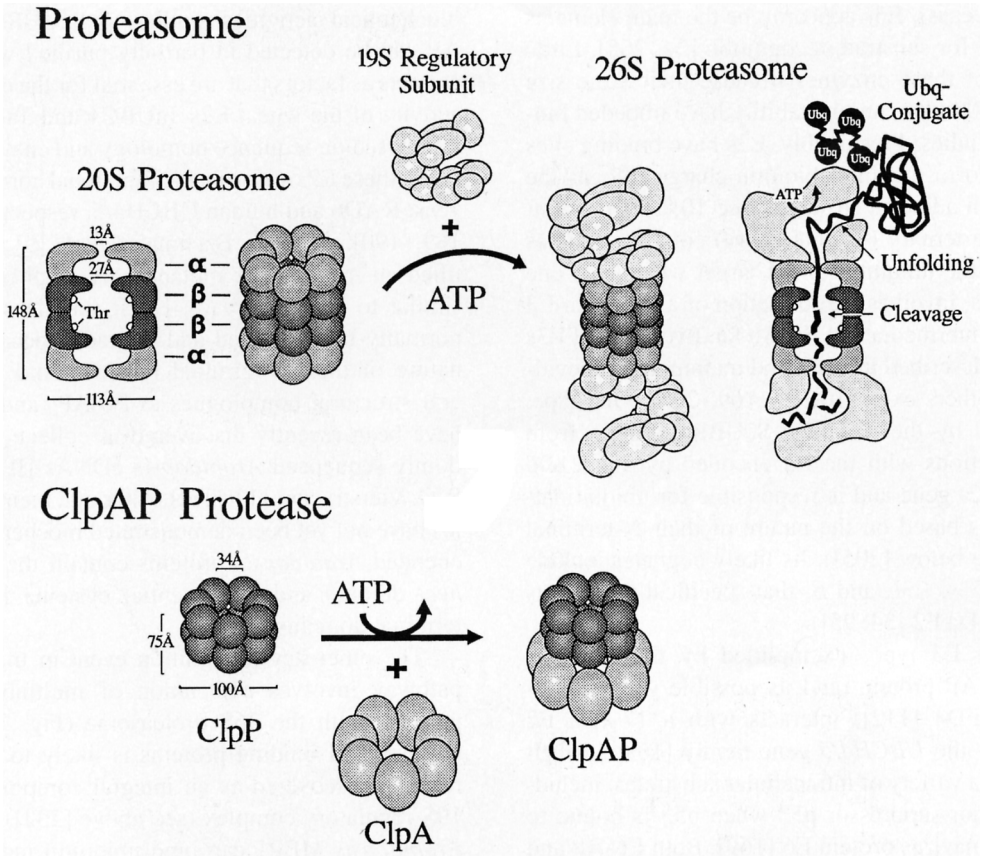


*Figure 4.* Proposed structures of the 20S and 26S proteasomes, involved in ubiquitin-dependent proteolysis, and the ClpAP protease. Structure of the 20S proteasome was determined by X-ray crystallography of that derived from the bacterium *Thermoplasma* [135]. Proposed structures of the 19S regulatory subunit of the 26S proteasome and ClpAP were created from electron microscopic images of the particles prepared from spinach and rat [193] or *E. coli* [61, 143], respectively. Details of the proteolytic complexes are described within the text. Thr, active-site threonine; Ubq, ubiquitin.

tial amino acid sequence dissimilarity within the core of different E2s (of up to 70%), they fold into a similar three-dimensional structure [36, 38]. Certain E2s also contain additional sequences within the core or extending beyond the N- or C-termini [109, 145]. Several of these additions allow E2s to interact directly with specific substrates (at least *in vitro*) suggesting that they play a role in substrate binding in the absence of an E3 [189, 200]. In fact, it has been shown that either transfer of natural C-terminal extension from one E2 core to another or addition of a synthetic protein-binding domain to an E2 core can be sufficient to confer E3 independence and appropriate substrate recognition [81, 189].

In yeast, genetic analysis has identified twelve E2 genes encoding eleven structurally different proteins (the exception being the closely related *ScUBC4* and *5* genes [34, 109]). A number of E2 genes have been discovered in plants as well. In *Arabidopsis*, seventeen E2 genes have been characterized to date encoding six different E2 types (*AtUBC1-17* [13, 68, 72, 190, 203]); four of these have counterparts in yeast. A cDNA encoding a seventh plant E2 type was recently isolated from tomato with homology to a 25-kDa mammalian E2 [31], but unrelated to any of those in yeast [S. van Nocker and R.D. Vierstra, unpublished]. Mutations in many of the yeast E2 genes lead to distinct phenotypes suggesting that the corresponding proteins ubiquitinate different substrates [34, 109]. For one target, the MAT$\alpha$2 repressor, multiple E2s work in concert suggesting that target specificity can be further expanded by various permutations of E2s forming heteromeric complexes [30].

In most cases, E3s appear to be the main elements responsible for substrate recognition [34, 205]. Little is known of these enzymes because their large size (100 to >300 kDa) and instability have impeded biochemical studies. Presumably, E3s have binding sites for both a corresponding ubiquitin-charged E2 and the target (or an adjacent structure [see 10, 169]). Formation of the ternary (or quaternary) complex enables transfer of the ubiquitin to the target which, for one class of E3s, involves the formation of a ubiquitin-E3 thiol-ester intermediate [170]. So far, five types of E3s have been described in yeast and mammals with evidence that others exist [10, 79, 169, 205]. One type, exemplified by the 225 kDa ScUBR1 protein from yeast, functions with the E2 encoded by the *RAD6* (or *ScUBC2*) gene and is responsible for ubiquitinating proteins based on the nature of their N-terminal residue (see below [205]). Its likely cognates include the rabbit E3s, E3$\alpha$ and $\beta$, that specifically interacts with a 14-kDa E2 [34, 95]).

Another E3 type, exemplified by the 100-kDa human E6-AP protein (and its possible yeast homologue ScUFD4 [112]) interacts with a 17-kDa E2 encoded by the *UBCH4/5* gene family [169, 170]. It recognizes a variety of intracellular substrates, including the tumor suppressor p53 when p53 is bound to the papillomavirus protein E6 [169]. Both E6-AP and ScUFD4 have a consensus ca. 30-residue C-terminal sequence, defined as the *hect* domain (for *h*omology to *E*6-AP *C*-*t*erminus), that includes a contextually conserved cysteine essential for ubiquitin transfer [104, 112, 170]. This *hect* signature was detected in a number of other proteins with previously unknown functions; subsequent biochemical studies on several of these showed that they have 'E3-like' activity [104]. Beyond the *hect* domain, the proteins share little sequence homology, suggesting that the rest of each molecule is involved in E2 specificity and/or the recognition of distinct substrates. Yeast RAD18 protein, required in postreplicative DNA repair, may represent a third type of E3 activity [10]. RAD18 associates with both the RAD6 E2 and single-stranded DNA and may help RAD6 ubiquitinate specific chromatin-associated proteins during the repair of single-stranded DNA gaps. This mechanism of *trans*-conjugation for RAD18 has been observed for ScUBR1 (and maybe E6-AP) as well [111, 169], indicating that E3s can also interact indirectly with their targets through association with other factors (nucleic acid or protein).

Several lines of evidence indicate that E3 counterparts to ScUBR1 and E6-AP exist in plants. First, biochemical activities similar to ScUBR1 and E6-AP can be detected in partially purified wheat germ extracts as factors that are essential for the conjugating activity of the wheat E2s TaUBC1 and TaUBC8 [71, 72]; based on sequence homology and enzymatic analyses, these E2s are probable functional homologues of yeast RAD6 and human UBCH4/5, respectively [169, 189, 190]. Second, Bachmair *et al.* [9] have identified an *Arabidopsis* mutant (*prt1-1*) phenotypically similar to yeast *Scubr1*$^-$; i.e. it stabilizes substrates normally ubiquitinated and degraded because of the nature of their N-terminal residue. And third, several structural homologues to E6-AP (and ScUFD4) have been recently discovered in collections of randomly sequenced *Arabidopsis* cDNAs [P. Bates and R.D. Vierstra, unpublished]. Although their E3 activities have not yet been demonstrated biochemically, the encoded *Arabidopsis* proteins contain the consensus *hect* domain and the essential cysteine in their C-terminal domains.

The other key recognition event in the ubiquitin pathway involves association of multiubiquitinated proteins with the 26S proteasome (Fig. 3). One of the essential binding proteins is likely to be MBP1, recently discovered as an integral component of the 19S regulatory complex (see above [202]). Although *Arabidopsis* MBP1 can bind ubiquitin monomers, it prefers multiubiquitin chains containing four or more ubiquitins [202]: a binding specificity that may explain the need for multiubiquitination prior to target degradation by the 26S proteasome [29]. It is possible that binding of chains to MBP1 not only promotes recognition of conjugates by the 26S proteasome, but also serves to tether ubiquitinated proteins to the 26S proteasome until the entire target is unfolded and degraded.

How MBP1 binds multiubiquitin chains is clear. That *Arabidopsis* MBP1 and its yeast and human homologues can recognize ubiquitin chains even following SDS-denaturation and adhesion to nitrocellulose indicates that the primary sequence of MBP1 and/or a highly stable secondary structure is probably involved [48, 202]. In solution, free MBP1 can act as a potent and specific inhibitor of ubiquitin-dependent proteolysis *in vitro*, presumably by competing for conjugates with 26S proteasome-associated MBP1 [49]. One model for binding of MBP1 to multiubiquitin chains proposes that a hydrophobic patch found twice within many MBP1 homologues associates with a repeated hydrophobic patch on the surface of multiubiquitin chains [14,202]. Nonetheless, it is likely that MBP1 does not work alone but in concert

with other 19S subunits. van Nocker *et al.* [204] have demonstrated recently that deletion of the yeast counterpart is not lethal and only impairs degradation of a subset of ubiquitin pathway targets.

*Ubiquitin-independent pathways*

In addition to the ubiquitin-dependent system, the cytoplasmic and nuclear compartments of plants and animals likely have other proteolytic pathways. Two may involve the 20S and 26S proteasomes by themselves. For example, degradation of ornithine decarboxylase (ODC) in animals is dependent on the 26S proteasome but independent of ubiquitin [150]. Targeting requires association of ODC with antizyme, a small protein that appears to facilitate docking of ODC with the 26S complex [150]. This precedent shows that the 26S proteasome can recognize short-lived proteins by signals other than ubiquitination. Ubiquitin-independent recognition conceivably could occur through direct interaction of substrates with the proteasome, indirect associations of substrates through other factors (e.g. antizyme), or interactions of substrates by a more generalized, ubiquitin-independent tagging mechanism (e.g. phosphorylation, methylation).

Another proteolytic system could involve the $Ca^{2+}$-activated neutral protease, calpain, found in a variety of vertebrates, invertebrates, and fungi [42]. While the exact functions of calpain is still unclear, it may be involved in the complete degradation of mature proteins as well as the limited proteolysis of preproteins. Two isoforms of this protease are known that differ in their $Ca^{2+}$ sensitivity. Both exist as a heterodimer between one of two distinct 80-kDa catalytic subunits that contain a cysteine-protease domain linked to a calmodulin-like $Ca^{2+}$-binding domain and a common 30-kDa regulatory subunit that contains another calmodulin-like domain [42]. $Ca^{2+}$ is required not only for activity but also for autoproteolytic activation of each subunit's proenzyme precursor. Although the presence of calpains has not been unequivocally demonstrated in plants, $Ca^{2+}$-activated proteases have been detected [162]. As of yet, no plants genes have been isolated with convincing homology to calpain subunits from either animals or fungi (R.D. Vierstra, unpublished).

*Protein degradation in organelles*

*Vacuoles*

Vacuoles are the largest organelles in plants, occupying as much as 90% of the total cell volume. Like yeast vacuoles, they contain a variety of hydrolytic activities including a number of proteases, hence they are often called the 'lytic compartments' of the plant cell [44, 139] (Fig. 1). In fact, these vacuolar proteases account for most of the proteolytic activity measured in plant extracts and thus, are likely responsible for one of the main technical problems associated with trying to purify plant proteins intact. A wide range of proteases have been detected including, endo- and exoproteases, amino- and carboxyl peptidases, and aspartic acid-, cysteine- and metallo- and serine proteases; some are commercially important, including papain (papaya), ficin (fig), and bromelain (pineapple) [12]. Most vacuolar proteases perform optimally at acidic pH (pH 3–6), a condition that exists within the vacuole *in vivo*. None have been discovered that are energy-dependent.

Despite the myriad of proteases present, the role of vacuoles in general protein breakdown is still unresolved. By analogy with animal lysosomes, it was originally proposed that plant vacuoles are responsible for degrading most cellular proteins, including those from the cytoplasm and chloroplasts which were thought to enter the vacuole primarily via autophagy [139]. However, (1) the subsequent identification of plant proteolytic pathways outside of the vacuole [25, 207], (2) the ability of yeast defective in major vacuolar proteases to degrade normally protein from other compartments [113, 194], and (3) the ability of plants to degrade intracellular proteins even when most vacuolar protease activities are inhibited [148] have led researchers to question this role and, in fact, suggests that vacuolar proteases contribute little to total protein breakdown in a typical plant cell.

Nevertheless, collective evidence does not rule out more specialized proteolytic roles for plant vacuoles. In fact, recent data suggests that plants contain two types of vacuoles, one with an acidic pH like the lysosome [221]. One well documented proteolytic function involves protein bodies, a specialized form of the vacuole responsible for the storage and mobilization of protein reserves during seed germination [59, 216]. During seed maturation, specific storage proteins are synthesized on rough endoplasmic reticulum (ER) and subsequently transported into these membrane-bound vesicles. The vesicles likely arise from fragmentation

of larger vacuoles. Proteins within these protein bodies are stable until germination, at which time specific proteases are synthesized *de novo* and transported into the vesicles to initiate proteolysis [16, 59, 216]. Some of the storage protein-degrading proteases are related to the cathepsin class of cysteine proteases, found in mammalian lysosomes [16, 99, 124]. This type of storage and mobilization is not restricted to seeds but can also be observed in leaves, seed pods, and seedling hypocotyls. Here, a small family of vegetative storage proteins are synthesized and sequestered in vacuoles during periods of high nitrogen availability and are subsequently degraded when the tissue becomes nitrogen-limited or senescent or when the stored amino acids are needed by sink tissues [185].

In addition to degrading storage proteins, vacuolar proteolysis likely serve other functions. Vacuolar proteases (and other hydrolytic activities) may help plants defend against pathogens, parasites, and herbivores by attacking the invader once the plant cell is lysed [18, 141]. Consistent with this role is the vacuolar location of various proteinaceous inhibitors to animal and fungal proteases [166]. Vacuolar proteases could act during the final stages of plant senescence by degrading any remaining cytoplasmic and organellar substrates after rupture of the vacuolar membrane. They may assist in the proteolytic processing of vacuolar zymogens. And finally, vacuolar proteases may help supply free amino acids during times of rapid growth, starvation, or stress [185, 194]. In addition to storage proteins, this breakdown could involve a variety of other vacuolar and cytosolic proteins.

If cytosolic targets are involved, stress-enhanced vacuolar degradation would necessitate active transport of proteins into the organelle. Although such a transport system has not yet been demonstrated in plants, an animal paradigm exists involving lysosomes, which are responsible for enhanced protein degradation during nutrient deprivation [50]. The pathway employs a cognate of the heat-shock 70 kDa protein family, PRP73, whose abundance in the cytosol increases about twenty-fold during starvation [32]. PRP73 binds to a group of cytosolic proteins that all bear a consensus motif, KFERQ, and then facilitates the ATP-dependent transport of the bound proteins into the lysosome where degradation commences [50]. About 20–30% of cytosolic protein in mammalian cells have this motif and thus are enlisted by this accelerated lysosomal degradation. It would be appealing to evoke a similar degradation system for plant vacuoles, especially during nitrogen deprivation and leaf and flower senescence.

*Chloroplasts*

Chloroplasts are protein-rich compartments in plants, containing up to 50% of the total cellular protein in photosynthetic tissue. As a result, much attention has been given to understanding how chloroplast proteins are degraded, especially during leaf senescence when much of the protein lost is of chloroplastic origin [43]. Chloroplast proteins were originally proposed to be degraded by vacuolar proteases [43] and more recently by the ubiquitin pathway [see 15, 207]. However, it now appears that neither mechanism is involved and that chloroplasts have variety of internal proteases, some of which require ATP [130, 137, 180] (Fig. 1).

One important chloroplast protease is related to the bacterial ATP-dependent protease Clp, first identified in *E. coli* [143]. Bacterial Clp is composed of two types of subunits: ClpP, a 21 kDa serine protease, and ClpA, a 81 kDa ATPase that uses ATP hydrolysis to activate ClpP and unfold protein substrates [143, 214]. Protein degradation requires the ATP-dependent assembly of two heptameric rings of ClpP subunits with a single ring likely composed of seven ClpA subunits [61, 119] (Fig. 4). Both the 20S proteasome and the GroEL chaperonin (involved in protein folding) also have a similar hollow barrel structure, suggesting that protein unfolding/folding is fostered by this three-dimensional arrangement [212].

Homologues to both *E. coli* ClpP and ClpA were first discovered in a variety of plants (including *Arabidopsis*, pea, tobacco, tomato, rice, and wheat) by DNA sequence homology [82, 86, 142, 147, 180] and since have been confirmed by functional assays of the encoded proteins [180]. Whereas, the plant ClpP protein is encoded by the chloroplast genome, the ClpA protein is encoded by the nuclear genome, synthesized in the cytoplasm, and transported in the chloroplast where it assembles with ClpP. In this way, the nucleus has the potential to tightly regulate chloroplast proteolysis by controlling the synthesis of ClpA regulatory subunit. Additional regulation may be accomplished by using alternate ClpA subunits. Recent evidence indicates that several types of ClpA proteins are present in bacteria [83], each of which may be differentially regulated and/or recognize distinct groups of substrates.

Besides ClpAP, chloroplasts have a variety of other proteases, including several neutral proteases [131], a prolyl endopeptidase [126], a stroma-located metal-

loprotease EP1 that may be involved in degrading Rubisco [23], and two proteases required for the removal of transit peptides from imported proteins [155]. None of the proteases for which clones are available appear to be encoded by the chloroplast genome, further underscoring the notion that most plastid proteolysis is accomplished with the help of nuclear-encoded enzymes. However, a maternally-inherited mutant has been characterized from *Oenothera* that blocks processing of chloroplast precursor proteins, indicating that the chloroplast does encode essential functions in this proteolytic process [110]. Another prominent bacterial protease that may be present in chloroplasts is protease La (encoded by the *LON* gene); a protease essential for degrading abnormal proteins in *E. coli* [75, 143]. La is an ATP-dependent serine protease assembled as an oligomer of identical 105-kDa subunits. Recently, a maize gene encoding a homologue of protease La was identified (W. Rapp and S. Barakat, pers. comm.). While the location of the encode protein remains to be determined, the initial translation product contains an N-terminal leader similar to the chloroplast transit sequence.

*Other organelles*

Currently little is known about how other plant organelles degrade proteins (Fig. 1). In animal cells, there is evidence that mitochondria, microbodies, and the ER and its connected secretory system have proteolytic pathways associated with the maturation and maintenance of each compartment [75, 123, 211]. In each case, degradation is highly selective. For example, rat mitochondria degrade 30–50% of total cellular protein within a hour of synthesis, with an even faster rate if the substrates are abnormal [47]. Animal mitochondria contain several protease activities; one is nuclear-encoded and appears related to the *E. coli* protease La [75].

Following translation, a substantial number of proteins enter the ER where they are extensively modified, assembled into complexes, and sorted *en route* to the Golgi, vacuole, various membranes, or apoplastic space. Proteolysis likely serves three main roles in the ER and its connected secretory system [123]. One is to remove unwanted normal proteins. A second is to dispose of improperly folded or assembled proteins. In animal, such dysfunctional proteins are retained in the ER (possibly by association of improper protein conformations with BiP, an ER-resident chaperonin [116]), where they are rapidly degraded by internal proteases (half-lives of 10–60 min) [123]. Similar retention and removal of abnormal proteins likely occurs in plant ER as well. For example, several maize mutants defective in endosperm development not only inhibit accumulation of the zein storage protein family, presumably by increased proteolysis, but also concomitantly increase the levels of the plant BiP chaperonin [19].

A third proteolytic role of the ER is to assist in protein maturation by proteolytically processing larger precursors. One example of this in plants may involve systemin, an 18-amino acid peptide hormone synthesized during defense response in tomato [169]. The active peptide is generated by proteolytic processing an inactive 200-amino acid precursor. Processing likely occurs in the secretory system, possibly by a subtilisin-type serine protease related to the yeast KEX2 protease [167]. KEX2 protease is an integral membrane protein of the Golgi and is responsible for generating a number of peptide hormones, including the yeast $\alpha$-factor mating pheromone, by cleaving at the carboxyl side of dibasic residues, Arg-Lys and Arg-Arg [11]. Plant genes encoding KEX2 homologues have recently been isolated from *Alnus*, *Arabidopsis*, and melon [163, 219].

Proteolysis is also required for the function of microbodies, especially when they differentiate into the various specialized forms during plant development. For example, the shift of glyoxysomes to peroxisomes in cotyledons requires both the import of enzymes involved in photorespiration and the selective removal of enzymes previously involved in lipid $\beta$-oxidation [56]. This proteolysis could be triggered by the import of specific proteases or the selective export of the unwanted proteins. The ubiquitin system has been implicated, at least in part, based on the recent discovery that genes essential for peroxisome biogenesis in the yeasts, *S. cerevisiae* and *Pichia pastoris*, encode E2s integrally bound to the cytoplasmic face of the peroxisomal membrane [40, 211].

## Functions of proteolysis

A picture is emerging, especially over the last decade, that protein degradation is an essential component in many aspects of plant growth, development, and environmental responses. Although most data on function have been obtained with organisms better suited to genetic manipulation (e.g. *E. coli*, yeast, and *Drosophila* [34, 97, 102, 109, 143, 151]), the conservation of many proteolytic pathways implies that the con-

clusions drawn from these models systems pertain to plants as well. Based on several lines of evidence, the ubiquitin pathway likely plays a particularly pervasive role in plants. First, expression studies with a number of plant species, using immunoblot analysis for protein or northern blot analysis or GUS-reporter histochemistry for mRNA, show that ubiquitin and many other pathway enzymes are present in most, if not all, plants cells [22, 27, 69, 92, 195]. Second, the pathway is important to normal plant development based on the severe morphological abnormalities induced in tobacco when a functionally impaired mutant of ubiquitin (Lys-48 to Arg) is expressed [8]. Third, a number of plant proteins are probable ubiquitin-pathway targets, judging from the innumerable conjugates that can be detected in extracts from various plant species [15, 22, 201, 209]. However, as in all other organisms, the identity of most of these targets remains to be discovered.

*Removal of abnormal/damaged proteins*

One of the first recognized functions of proteolysis is its role in cellular housekeeping. Abnormal proteins continually arise by a variety of mechanisms including mutations, biosynthetic errors, spontaneous denaturation and free radical-induced damage and can be accelerated by environmental stresses such as heat shock, desiccation, high-fluence light, disease, nutrient deprivation, and exposure to heavy metals or amino acid analogues [65, 143, 207]. Accumulation of aberrant proteins not only squanders valuable supplies of plant nitrogen but also can disrupt the integrity of subcellular compartments and various macromolecular structures should they accumulate to sufficient levels. Removal of abnormal proteins is especially important to plants where cell division rates are much too slow to passively reduce intracellular concentrations. In many situations, damaged proteins can be repaired or refolded with chaperonins helping to restore native conformations [65]. However, for some proteins or in some situations where the levels of abnormal protein become too high (e.g. heat shock), proteolysis is an important solution. The chloroplast encoded 32-kDa D1 protein, one protein in the core of the photosystem II reaction center complex, is one such example. To maintain photosynthetic electron flow under high light stress, chloroplasts are continually replacing damaged D1 with functional counterparts [140].

Each major compartment in plants must have mechanisms for degrading abnormal proteins (Fig. 1). In the cytoplasm and nucleus, the ubiquitin pathway is an important route [109]. Consistent with this role, perturbations in the ubiquitin pathway heighten the sensitivity of tobacco and yeast to amino acid analogues and other conditions that exacerbate protein denaturation [8, 109]. In yeast, the ScUBC4/5 E2s are essential for removing abnormal proteins [109]. Their plant homologues include the wheat TaUBC8 and *Arabidopsis* AtUBC8-12 E2s [71, 72]. TaUBC8 works with a wheat E3 designated E3$\gamma$, at least *in vitro*, which may be a plant homologue of human E6-AP [71, 169]. The TaUBC8/E3$\gamma$ pair is responsible for most of the ubiquitin-conjugating activity in wheat germ extracts, implying that this E2/E3 pair could play a prominent ubiquitinating role *in vivo* [71]. It is unclear which proteases remove abnormal chloroplast proteins. By analogy with bacteria, ClpAP (and possibly protease La) is a probable candidate given its essential role in removing abnormal proteins in *E. coli* [75, 143].

A variety of conditions the exacerbate protein denaturation also activate proteolytic pathways. For example, genes encoding ubiquitin and a number of conjugating enzymes are activated in plants, yeast, and animals by desiccation, heat shock, heavy metals, and infection [33, 66, 109, 207]. In bacteria, both the La and ClpAP proteases genes are heat shock-inducible [74, 143] whereas, in *Arabidopsis*, the ClpA gene is drought-inducible [122]. It should be noted that the stress activation of specific proteolytic genes may not be universal among plant species; although heat shock induction of ubiquitin genes is strong in maize [33], potato [62] and tobacco [66], it occurs weakly, if at all, in *Arabidopsis* [22]. Whether this implies that the ubiquitin system is not involved in the heat shock response in some plants (e.g. *Arabidopsis*) or is already at sufficient levels in these plants to handle the stress is unknown. Activation of proteolytic pathways is also intimately tied to activation of other stress-related proteins (e.g. chaperonins) involved in damage control [65]. Even in the absence of stress, chaperonin synthesis in bacteria, animals, and plants can be enhanced either by inactivating proteolytic pathways that remove abnormal proteins or by overloading proteolytic pathways with denatured protein [3, 74, 75, 109, 127]. Enhanced accumulation of BiP chaperonin in the ER of maize endosperm mutants defective in zein synthesis is a prime example in plants [19].

Recognition of incorrectly folded proteins probably involves general properties of the target in ways that allows many types of denatured proteins to be detected by chaperonins or proteolytic pathways regardless of

their amino acid sequence. The most likely determinant would be an increased exposure of hydrophobic surfaces normally buried in native conformations [65]. This exposure could either provide binding sites for chaperonins involved in refolding/unfolding, recognition sites for proteolysis, or compel proteins to spontaneously aggregate. Aggregation could provide a convenient mechanism to sequester abnormal proteins prior to catabolism [143, 207].

For those aberrant proteins translated from truncated mRNAs, Sauer and coworkers recently discovered that *E. coli* has developed a highly sophisticated mechanism for their removal [117]. Because the termination codon is essential for releasing the nascent polypeptide from ribosome-bound tRNA once translation is complete, incomplete polypeptides remain bound to and thus stall ribosomes. This stalling triggers the association of the 10Sa RNA, a tRNA-like RNA containing a charged Ala, with the ribosome. The ribosome subsequently transfers the nascent polypeptide chain onto the bound Ala moiety. After the truncated mRNA is released, the ribosome switches to translating an internal sequence within the 10Sa RNA encoding the nonapeptide NDENYALAA, which is then followed by a termination codon. Once the chimeric polypeptide is released, the *E. coli* *t*ail-*s*pecific *p*rotease, Tsp, recognizes the C-terminal addition and rapidly degrades the tagged protein [117, 182]. This translational switch serves two purposes: it facilitates release of defective mRNAs and marks potentially harmful proteins for breakdown. Tsp protease, which can also degrade normal proteins, has homologues in various Gram-positive and Gram-negative bacteria and animals [4, 117, 182]. In the cyanobacterium, *Synechocystis*, a Tsp homologue is responsible for proteolytically processing of the photosynthetic D1 protein [4]. An intriguing possibility is that a Tsp relative also exists in chloroplasts.

*N-end rule pathway*

Another group of aberrant proteins are those that are improperly processed or become mis-localized. In many cases, these errors generate polypeptides with N-termini atypical of proteins normally localized to the compartment. For example, whereas the N-termini of most cytoplasm and nuclear proteins are either Met, Gly, Ala, Ser, Thr, or Val and/or are N-acetylated [5], those that are incorrectly processed or from the ER and other organelles will not likely have these termini [5, 7]. Varshavsky and co-workers have discovered in yeast, mammalian cells, and *E. coli*, that these foreign N-termini are universally exploited as recognition sites for an N-end rule pathway that removes such unwanted proteins [7, 205]. However, this degradation scheme is not limited to aberrant proteins as the levels of several normal proteins are regulated by this method as well [136, 205].

In the cytoplasm and nucleus of animals and yeast, the N-end rule pathway involves components within the ubiquitin system and contains a hierarchy for amino acid recognition [205]. In yeast, recognition of inappropriate N-termini is accomplished by the RAD6 (or ScUBC2) E2 working in concert with the E3$\alpha$, ScUBR1 [205]. RAD6/ScUBR1 directly bind to polypeptides with Arg, Lys, or bulky hydrophobic N-termini and hence these amino acids are called primary destabilizing residues within the N-end rule. Asn and Gln are called tertiary destabilizing amino acids because they first must be deamidated by an N-terminal deamidase to generate secondary destabilizing residues, Asp and Glu. Arg is added to these acidic N-termini by an arginyl transferase, using Arg-tRNA as the donor, to then generate the primary destabilizing Arg N-terminus that is finally recognized by ScUBR1.

Components of the N-end rule pathway have been identified in plants. The *Arabidopsis* E2s encoded by the *AtUBC1-3* genes appear to be the RAD6 counterparts based on amino acid sequence similarities and an analogous requirement for E3$\alpha$ *in vitro* [72, 190]. The *Arabidopsis* mutant (*prt1-1*) that is incapable of degrading proteins with Phe N-termini may affect the plant counterpart to ScUBR1 [9].

In *E. coli*, the N-end rule pathway is executed by ClpAP [197]. Its N-end rule hierarchy appears to be more restricted than those in yeast and animals and includes just bulky hydrophobic residues (e.g. Phe, Leu, Tyr, Trp) and Arg and Lys as destabilizing residues [197, 205]. Only bulky hydrophobic residues are primary destabilizing residues; they are recognized directly by ClpAP and degraded. Lys or Arg are secondary destabilizing residues. Leu is added to these N-terminal residues by a Leu/Phe transferase to generate N-termini with the primary destabilizing residues Leu. Given the presence of ClpAP homologues in chloroplasts [82, 180], it is likely that chloroplasts have the accompanying N-end rule pathway as well. In this way, chloroplast ClpAP could assist in the removal of incorrectly processed cytoplasmic precursors, as well as in the removal of misfolded proteins.

*Supply of amino acids*

Even though plants can synthesis all amino acids *de novo*, a substantial portion of new proteins are derived from recycled amino acids [44, 103, 207]. These amino acids can be generated from the proteolytic housekeeping of abnormal and unwanted proteins, or they can be derived from specialized versions of normal proteins whose sole purpose is to store amino acids. Both seed storage proteins and vegetative storage proteins are examples of this latter group [59, 185, 216]. Degradation of vegetative storage proteins, and possibly other proteins, are accelerated during nitrogen deprivation suggesting that plants have signalling pathway(s) linking the supply of free amino acids to the rate of intracellular proteolysis [185]. By analogy with animal and bacterial starvation responses, these pathways could monitor levels of uncharged tRNAs as an indirect measure of low amino acid supplies [174].

Extracellular proteolysis can also provide an important source of amino acids in plants. This process not only necessitates the export of proteases into the apoplast but also the existence of amino acid and peptide transporters for import of proteolytic products. Both of these components have been detected in a variety of plants [25,59, 186]. For example, extracellular proteolysis of endosperm storage proteins in cereal seeds provides most of the amino acids used by the developing embryo [59]. This degradation is accomplished by secretion of proteases from scutella epithelia and aleurone layers. During barley seed germination, a complex of proteases are secreted, including two cysteine proteases, EPA and EPB, with specificity toward the hordein storage proteins of the endosperm [124]. Amino acid and peptide transporters, needed to import proteolytic products, have been detected in plants as well; one from *Arabidopsis* is a member of a membrane-transport protein family also found in yeast and animals [see 186]. In a special case of extracellular proteolysis, carnivorous plants have developed a highly sophisticated morphology to trap, digest, and absorb insects as an additional source of amino acids and nitrogen [114].

*Control of enzymatic pathways*

One of the central functions of proteolysis is to help regulate metabolism by directly controlling the levels of key enzymes. Global use of this strategy first became evident from a survey of enzymes responsible for catalyzing either the first or rate-limiting step in various metabolic cascades; all had short half-lives [44, 77, 103]. In some cases, the short half-life is constitutive, whereas in others, it is developmental or environmentally regulated or induced by limiting substrate or excess product. Such instability allows cells to control the metabolic flux through a pathway simply by attenuating synthesis of a crucial enzyme and then allowing degradation to rapidly reduce its levels [77]. The advantage of degradation over other methods of metabolic regulation is its speed (half-lives of minutes) and that elimination of the protein negates any possibility that the enzyme can be reactivated inappropriately. Examples of such short-lived plants proteins include: NADPH protochlorophyllide oxidoreductase, fructose bisphosphatase, ATP sulfurylase, HMG-CoA reductase, ornithine decarboxylase, squalene synthetase, and phenylalanine ammonia lyase which catalyze important or committed steps in chlorophyll production, carbon, and sulfur metabolism, and sterol, spermine, isoprenoid, and lignin biosynthesis, respectively [25, 44, 103, 207]. Such proteolytic regulation can also encompass whole metabolic pathways. For example, the entire $\beta$-oxidation pathway is removed from the cotyledons of dicotyledonous plants during the transition from glyoxylate- to photosynthate-dependent growth [56].

Nitrate reductase (NR), which catalyzes the first step in the conversion of nitrate to ammonia, is one of the best studied plant examples of metabolic regulation by proteolysis [41]. By a combination of mechanisms, NR activity increases in the presence of nitrate and light and decreases in their absence. Darkness or removal of nitrate not only down-regulates NR gene transcription but also induces the rapid, but reversible inactivation of the NR protein. In spinach and maize, NR is inactivated by phosphorylation of the protein [41, 115]. This inactivation is then followed by rapid degradation of the NR polypeptide. Although a connection between phosphorylation and NR proteolysis has not yet been made, it is conceivable that the added phosphates aid in the recognition of inactive NR by specific proteases [41]. As NR is a cytosolic enzyme, participation of the ubiquitin pathway is plausible. A region of ca. 50 amino acids near the N-terminus of tobacco NR appears to be required for its inactivation and/or proteolysis [154].

Protein breakdown also controls the levels of ACC synthase, the enzyme which catalyzes the first step in ethylene biosynthesis, the conversion of *S*-adenosyl-L-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC). This cytoplasmic protein has an extremely

short half life (20–120 min depending of the tissue or plant species [118]). Although the mechanism of ACC breakdown is unknown, it does appear to be energy-dependent as uncouplers of oxidative phosphorylation block protein loss [120]. Studies with both protein kinase and phosphatase inhibitors suggest that ACC synthase degradation, like that of NR, is regulated by phosphorylation/dephosphorylation of the protein [183].

With respect to multi-subunit complexes, proteolysis also assists in correcting the inappropriate stoichiometry of subunits and in maintaining correct enzyme/cofactor ratios [207]. Rubisco is an example of a multi-subunit enzyme whose stoichiometry is corrected in this way. In the absence of the chloroplast-encoded large subunit, unassembled nuclear-encoded small subunit is rapidly degraded upon import into the organelle [172]. Similarly, chlorophyll *a/b*-binding proteins and plastocyanin are rapidly catabolized in the absence of their respective cofactors, chlorophyll and $Cu^{2+}$ [145, 149]. The stoichiometric accumulation of a number of other chloroplast and mitochondrial enzyme complexes may be corrected in a similar fashion to help overcome a lack of precise coordination between the organelle and nuclear genomes. How the proteolytic machineries recognize unassembled subunits is unclear, but because many incompletely assembled complexes are conformationally unstable, they may be detected by the same general features as are abnormal proteins.

*Control of various cell regulators*

In addition to controlling many metabolic pathways, short-lived proteins also play crucial roles in various regulatory processes, including signal reception and transduction, homeosis, transcription, and cell growth and division [34, 95, 97, 205, 207]. In animals and yeast, a growing number of such regulators appears to be specifically degraded by the ubiquitin/26S proteasome pathway. Examples include transcription factors cJUN, cFOS, MOS, GCN4, the p53 tumor suppressor, V(D)J recombination activator protein RAG2, components of the NF$\kappa$B transcriptional complex, the yeast G-$\alpha$ protein Gpa1, and the MAT$\alpha$2 repressor involved in yeast mating-type switch [34, 97, 125, 129, 136, 153, 199, 205]. The yeast STE2 plasma membrane receptor is also conjugated with ubiquitin upon $\alpha$-factor induction, but degradation appears to take place in the lysosome/vacuole [96]. Various other cell surface receptors are ubiquitinated upon ligand engagement, including the platelet derived growth factor receptor, T-cell antigen receptor, and the immunoglobulin E receptor, but whether this ubiquitination targets the receptors for degradation has not yet been established [34, 101]. In addition to those regulatory proteins for which direct experimental evidence exists, a whole host of other regulatory proteins are predicted to be short-lived based on the phenotypic consequences of specific proteolytic defects. For example, mutations in specific components of the ubiquitin pathway affect DNA repair, peroxisomal biogenesis and protein translocation into the ER in yeast [40, 97, 109, 211], or alter eye cell fate, neuronal development, and cell proliferation in *Drosophila* [102, 138, 151]. These restricted phenotypes suggest that the mutations block the degradation of specific short-lived, but as yet unidentified, regulatory proteins.

In several situations, the chain of events responsible for regulatory protein degradation by the ubiquitin system has been partially deciphered and the domains within the target proteins responsible for their short half-life have been defined. Degradation of the yeast MAT$\alpha$2 repressor requires several E2s (ScUBC4/5, 6, and 7) and involves at least two different recognition domains within the repressor protein [30]. Mammalian c-JUN requires a 27 amino acid sequence near the C-terminus of the protein for ubiquitination, but any lysine(s) can serve as the attachment site [199]. Howley and coworkers have shown that p53 degradation can be accelerated in Hela cells by trans-conjugation. In this case, a third component (E6) provided by the various 'high risk' papillomaviruses promotes contact of p53 with the E6-AP E3 leading to the enhanced ubiquitination of p53 [169].

An intriguing example of proteolytic regulation is the activation of NF-$\kappa$B, a human transcriptional activator involved in the defense response [158, 198]. It is synthesized as a 105-kDa precursor (p105) that is processed into a 50-kDa mature form (p50) by proteolytically removing the C-terminal half of the molecule. p50 resides in the cytoplasm under non-stressed conditions as a ternary complex with p65 (or RelB) and I$\kappa$B, an inhibitory protein that masks the nuclear localization signal of the p50/p65 heterodimer. Activation by a number of defense signals, such as tumor necrosis factor $\alpha$, triggers the selective destruction of the I$\kappa$B subunit; the rest of the NF-$\kappa$B complex (p50/p65) then enters the nucleus to transcriptionally activate a number of defense related genes. Both the processing of precursor p105 and the removal of I$\kappa$B require ubiquitin conjugation and the 26S proteasome [158, 198].

Thus, the ubiquitin pathway can not only selectively remove polypeptides from a multi-subunit complex but can also selectively degrade a limited part of a single polypeptide. Initial studies indicate that selective ubiquitination of IκB requires phosphorylation of the inhibitor at either one of two adjacent serines [21, 198].

*Phytochrome A*

The best studied short-lived regulatory protein in plants is phytochrome A, a member of a morphogenic photoreceptor family involved in light perception [208]. Phytochromes are dimeric cytoplasmic proteins, with each subunit consisting of a linear tetrapyrrole chromophore covalently linked to a ca. 120-kDa polypeptide. They regulate photomorphogenesis by switching between two photointerconvertible forms, a red light-absorbing from Pr that is biologically inactive, and a far-red light-absorbing from Pfr that is biologically active. Upon conversion to Pfr, phytochromes initiate a diverse array of physiological and developmental responses that allow plants to optimize reception of photosynthetic light and to coordinate their life cycle with daylength. All other members of the phytochrome family are expressed at low levels and are stable both as Pr and Pfr. However, phytochrome A is unique in that it is highly expressed in young seedlings and while stable as Pr ($t_{1/2} > 100$ hr), it is rapidly degraded once converted to Pfr ($t_{1/2}$ ca. 1 hr) [208]. The short half-life of phytochrome A Pfr helps seedlings adapt to continually fluctuating light conditions as they grow through the soil by removing previous light signals stored as Pfr.

Shanklin *et al.* [178] investigated the possible involvement of the ubiquitin pathway in phytochrome A degradation in oat and found that soon after Pfr formation, the chromoprotein becomes rapidly ubiquitination *in vivo*. Like other targets of the ubiquitin pathway subsequently examined, ladders of ubiquitin-phytochrome conjugates were evident following SDS-PAGE, consistent with the addition of multiubiquitin chains of various lengths [178, 179]. Although a direct link between ubiquitination and Pfr degradation remains to be made, various kinetic analyses present a strong case for ubiquitin's involvement: (1) Pfr-induced ubiquitination and degradation could be observed in a variety of plant species, both monocot and dicot [106]; (2) the levels of phytochrome ubiquitin conjugates directly correlated with the extent of degradation [105, 106, 178]; and (3) ubiquitin-phytochrome conjugates were turned over much more rapidly than Pfr, consistent with the kinetics expected for a degradation intermediate [105, 106]. One interesting aspect of Pfr degradation is that both ubiquitination and breakdown follow a rapid ($t_{1/2}$ ca. 2 s at 25 °C), energy-dependent aggregation of Pfr in the cytoplasm [208]. The function of this aggregation is unknown but it may help quickly inactivate excess Pfr by sequestering it in a form that is amenable to slower proteolytic destruction.

Selective ubiquitination of phytochrome A could involve specific structural differences and/or differential aggregation between the Pr and Pfr forms [208]. The extreme C-terminus of phytochrome A appears to be required as its removal stabilizes the chromoprotein as Pfr [R.C. Clough and R.D. Vierstra, unpublished]. Interestingly, this sequence is highly divergent between phytochrome A and the other more stable phytochromes [208]. Initial studies have also implicated an internal domain as a multiubiquitin chain attachment site (residues 742–790) [179]. However, substitution of the invariant lysines in this domain to arginines does not affect the rate of Pfr degradation, suggesting that ubiquitin attachment may not be restricted to this region [K. Lohman and R.D. Vierstra, unpublished]. Like most other natural substrates of the ubiquitin pathway, the E2/E3 pair involved in Pfr recognition has not been identified.

*Other regulatory proteins in plants*

The levels of a variety of other plant regulatory proteins are also likely to be controlled by proteolysis but at present, few have been analyzed at this level [207]. One potential example is the *Arabidopsis* homeodomain protein, SHOOTMERISTEMLESS (STM), required for shoot meristem formation [134]. The STM transcript is expressed in the meristem and then rapidly disappears as leaf primordia emerge implying that the protein is only needed within a restricted window of differentiation. A similar fate may befall APETALA3, a floral homeodomain protein essential for early specification of petal and stamens [107]. A number of proteins whose expression is induced by auxin have extremely short half-lives [1]. These data in combination with the discovery that one auxin-insensitive mutant, *axr1*, affects a protein with some amino acid sequence similarity to E1s [128], suggests that the ubiquitin pathway and auxin responses are intertwined. Proteolysis of a key regulatory protein also may be involved in the wound response of tomato as the response can be

also be induced *in vivo* by the aminopeptidase inhibitor bestatin [168].

*Timing of the cell cycle*

To ensure correct progression through mitosis and meiosis, cells have adopted elaborate timing mechanisms and checkpoints to coordinate DNA replication, chromosome pairing and segregation, and cell division [108, 152]. Recent work on the cell cycle in fission and budding yeasts, various metazoans, and mammals has shown that correct traversal requires the timed proteolytic removal and replacement of key regulatory proteins [34, 97, 152]. In fact, proteolysis regulates a number of important checkpoints including: entry in S phase (DNA replication) from G1, progression through S, entry in M phase (mitosis), completion of anaphase, and the exit from mitosis [152].

Two types of proteins that must be degraded for correct progression through the cell cycle bind to and alter the activity of the cyclin-dependent kinase (CDK), the master switch of the cycle [152]. They are the S-phase and M-phase cyclins, which are positive regulators originally discovered because their oscillating levels coincided with the cell cycle [152], and a CDK inhibitor ($p40^{SIC1}$ in yeast [173] or p27 in mammals [157]). Initial degradation of CDK-inhibitor derepresses CDK which in turn initiates passage across the G1 to S boundary. Sequential accumulation of various cyclins then promotes various CDK activities. These activations not only initiate specific steps in G1, S, G2 and M phases but also promotes the rapid degradation of the associated cyclins [152]. In this way, a wave of cyclins is created, signaling that one checkpoint in the cell cycle has been completed and that the next step can be attempted. In addition to cyclins and the CDK inhibitor, data suggest that a third protein must also be degraded for cells to initiate anaphase and may be required for the release of sister chromatids [98]. Finally, proteolytic loss of all cyclins at the end of mitosis prevents inappropriate entry into another round of cell division until the cell is ready [152].

In yeast and animal cells, removal of the CDK inhibitor and various cyclins (both S- and M-phase-specific) requires the ubiquitin-dependent proteolytic pathway [73, 157, 173, 177]. In fact, it was observed long before the discovery of cyclin and CDK inhibitor degradation, that one consistent phenotype of mutants in E1 and several 26S proteasome subunits is cell cycle arrest [70, 80, 109]. In yeast, degradation of the CDK inhibitor $p40^{SIC1}$ requires the E2, encoded by the *CDC34* (or *ScUBC3*) gene [173], whereas degradation of the different cyclins require the E2s either encoded by the *CDC34* or the *ScUBC9* genes [177, 218]. Other factors (possibly E3s) are also involved in cyclin ubiquitination and appear to assemble into a large complex [121]. The S- and M-phase cyclins are degraded by different mechanisms [177, 218]. M-phase cyclins contain a specific targeting signal (called the destruction box) for ubiquitination; transfer of this box to other proteins is sufficient to induce rapid breakdown of the recipient protein in a cell cycle-specific manner [73]. Preliminary data suggest that degradation of S-phase cyclins requires their CDK-dependent phosphorylation [218].

Given that homologous mechanisms of cell cycle control exist in fungi and animals, we expect that the plant cell cycle is also regulated by protein degradation. Plant counterparts to cdc28 kinase and cyclins have been isolated [108]. However, no components specifically involved in the ubiquitination of cell-cycle factors have yet been detected. Transcripts encoding ubiquitin and several 20S proteasome subunits accumulate in proliferating *Arabidopsis* and tobacco cell cultures [66, 69]. Whether this activation indicates a direct role of the pathway during cell division or an indirect role by providing constituents essential to actively growing cells in not known.

*Programmed cell death*

One of the natural consequences in the development of multi-cellular organisms is the timed disintegration of specific cells [57]. This can be confined to single cells or small regions or can occur on a massive scale and involve whole organs. Examples in plants include leaf, flower and ovary senescence, fruit ripening, xylem and periderm maturation, petiole abscission, programmed abortion of organ primordia in unisex flowers, tapetum and stomium degeneration in anthers, valve dehiscence in seed pods, and the hypersensitive response during pathogen invasion [25, 85, 207]. A number of genetic and pharmacological studies indicated early on that cell disintegration is a highly controlled, complex process initiated by both intrinsic and extrinsic signals. Programmed cell death generally involves the activation of both nucleases and proteases to efficiently degrade the resident nucleic acids and proteins [25, 57, 146]. Presumably, this catabolism economizes the loss of nitrogen and carbon by exporting them to areas of growth or storage. This recycling is most evident

during leaf senescence where up to 70% of the total leaf protein can be retrieved [43, 196].

Leaf, flower, ovary, and fruit senescence has been intensively studied because they represent important agriculture problems. Onset of senescence is controlled, in part, by cytokinins and gibberellins and involves activation of a number of proteases [43, 84, 94, 196]. Several of these proteases have been isolated, including *Arabidopsis* SAG2 and SAG12 [94, 133] and pea TPP [84], which are cysteine proteases whose transcripts dramatically increase in senescing leaves and ovaries, respectively. ClpAP mRNA levels also rise during *Arabidopsis* leaf senescence implicating ClpAP in the senescence-induced loss of chloroplast proteins [S.-S. Gan and R. Amasino, unpublished]. The ubiquitin pathway may also be involved. Both ubiquitin and ubiquitin conjugates have been shown to increase in daylily flower senescence [39] whereas perturbation of the ubiquitin pathway accelerates leaf senescence in tobacco [8].

Xylogenesis has been recently studied as an example of a programmed cell death process restricted to individual plant cells. One model system involves the hormone-induced *in vitro* differentiation of *Zinnia elegans* mesophyll cells into tracheids. During *Zinnia* xylogenesis, the accumulation of several proteases has been observed [46, 220]. Ubiquitin has also been implicated in xylogenesis. In intact *Coleus blumei* stems, increased levels of immunodetectable ubiquitin was associated with regions of newly differentiated xylem [35]. Using promoter-GUS fusions, Thoma *et al.* [195] found enhanced expression of a number of E2 genes in differentiating *Arabidopsis* vascular tissue. And finally, marked abnormalities in tobacco vascular tissue can be induced by expressing a non-functional ubiquitin [8].

## Applications in biotechnology

In addition to its role in cell physiology, proteolysis also has important ramifications in attempts to improve crop plants through genetic engineering [100]. Here, manipulations of proteolysis can not only enhance the accumulation of foreign proteins intended to confer beneficial traits but also may be used to repress accumulation of unwanted endogenous proteins that interfere with important agronomic processes. In fact, several strategies to enhance or repress protein accumulation by proteolytic approaches have been developed in the past few years [see 81, 100, 205].

One of the obvious problems when attempting to ectopically express proteins to high levels is that plants, like other organisms, often recognize foreign proteins and degrade them rapidly. Thus, even when all other transcriptional and post-transcriptional processes are optimized, proteolysis can still be a major barrier to adequate accumulation [100]. As a result, new emphasis has been placed on understanding the factors that regulate protein stability and on developing methods to interfere with the responsible proteases. In several cases, protein turnover is controlled by short amino acid sequences (e.g. cyclin destruction box, N-terminal residue, KFERQ [73, 50, 205, 218]). Increased stability (and thus increased accumulation) can be engineered, in some cases, simply by removing these instability domains. For example, removal of the destruction box from M-phase cyclins has been shown to stabilize cyclins and permanently induce repetitive rounds of the cell cycle in *Xenopus* oocyte extracts [73]. The recent identification of essential proteolytic pathways also offers the potential to use pharmacological and genetic strategies to inactivate the interfering proteases. In this regard, the newly discovered inhibitors of the 20S and 26S proteasome may be useful in preventing plant senescence processes that require these proteolytic complexes [49, 58, 165]. A genetic approach has been successful in enhancing protein production in *E. coli* and yeast with the disruption of the ClpAP and La proteases [75, 143] or vacuolar proteases [113] and the ubiquitin pathway [109, 205], respectively. However, comparable mutants in plants are not yet available.

In an unusual case, ubiquitin itself has been exploited to augment protein accumulation. This approach, first developed by Butt and colleagues [24, 55], involves expressing proteins as translational fusions to the C-terminus of ubiquitin. It was based on the knowledge that (1) poorly expressed proteins often can be stabilized by fusion with a highly stable protein, (2) ubiquitin is a highly stable protein, and that (3) natural ubiquitin C-terminal fusions exist. In yeast and *E. coli*, expression of ubiquitin-fused proteins was dramatically enhanced; the accumulation of some recalcitrant proteins were increased over 200-fold [24, 55]. Furthermore in several cases, the protein products were found to be more intact and active than their nonfused counterparts. Whereas the engineered products remained as ubiquitin fusions when expressed in *E. coli* [24], in yeast they were correctly processed following the C-terminal Gly-76 of ubiquitin by endogenous ubiquitin $\alpha$-amino hydrolases to release both

the fused protein and ubiquitin in intact forms [55, 205] (Fig. 3).

A similar fusion strategy has been shown to work in plants as well, and can result in significant increases in protein accumulation [63, 100]. As in yeast, these fusions are rapidly processed (possibly co-translationally) to yield non-fused products. Likewise, the plant hydrolases appear capable of processing many types of synthetic ubiquitin fusions provided that Pro is not the first residue following ubiquitin $Gly^{76}$ (D. Hondred, J. Walker, and R.D. Vierstra, unpublished). In addition to enhancing protein accumulation, this method also permits the synthesis of proteins/peptides with N-termini besides Met.

Proteolysis can also be used as a strategy to remove unwanted proteins. One approach involves re-engineering proteins to enhance their recognition by proteolytic pathways. In several cases, the addition of instability domains has been exploited to convert otherwise stable proteins into ones that are rapidly degraded [73, 117, 199, 205, 218]. In a specialized case, Dohmen *et al.* [52] appended a temperature-sensitive domain for degradation thus creating a fusion protein with a turnover rate that could be environmentally accelerated. An alternative approach could involve re-engineering proteolytic pathways to rapidly degrade stable, unmodified proteins. Gosink and Vierstra [81] recently demonstrated the potential of this approach with respect to the ubiquitin pathway. They showed that E2 target recognition could be redefined *in vitro* simply by engineering E2s with appropriate protein-binding domains fused to their C-termini. The binding domains could originate from naturally occurring proteins that interact with the target or be artificial peptides selected by their binding affinity [81]. Addition of these binding domains facilitated *in vitro* ubiquitination of the target which could then lead to the ATP-dependent degradation of a normally stable protein. If successful *in vivo*, this targeted proteolytic approach could provide benefits not offered by the genetic methods commonly used to attenuate protein accumulation (e.g. antisense and gene silencing). These include: (1) its catalytic nature, (2) the fact that neither the target protein nor its corresponding genes need be altered, (3) the ability to selectively recognize individual proteins or a whole family of related proteins by choosing the appropriate recognition site for E2 binding, (4) and the ability to target proteins not encoded by the host cell [81]. The latter benefit could allow plant cells to be 'preimmunized' against pathogenic invasion by targeting key pathogen proteins for destruction.

## Conclusions

As can be seen from the wealth of data accumulating rapidly over the past few years, the field of protein degradation in plants is entering an exciting period. We are beginning to realize that proteolysis is more complex than previously understood, pervades a multitude of cellular processes, and provides cells with a number of creative strategies for regulation. Multiple degradative systems are now known to operate within plants, with several delineated by compartmental boundaries. The constellation of identified plant proteases is growing exponentially. In some cases, we now understand why proteolysis requires energy and where it is consumed, how proteolysis is sequestered from other cellular processes, why proteolysis does not generate large peptide intermediates, and how proteins are recognized and targeted for degradation.

Converging information from bacterial, animal, and plant systems show that the organization of proteolytic systems and their methods for detecting protein targets are frequently conserved, thus allowing information derived from one kingdom to be potentially useful in another. Examples include the detection of bacterial ClpAP [82, 180] and La protease [75] homologues in plants, respectively, and the detection of ubiquitin and the 20S proteasome in some archaebacteria [135, 217] and eubacteria [38, 192]. Three fascinating themes have emerged. One is the use of peptide tags as signals for degradation; these include the addition of ubiquitin to internal Lys residues [34, 95], N-terminal arginylation in the N-end rule [205], and C-terminal addition of the AANDENYALAA tag specific for the bacterial Tsp protease [117]. The second is the use of oligomeric barrel-shaped structures in the folding/unfolding and/or degradation of proteins [61, 143, 212] (Fig. 4). Presumably, this arrangement protects other cytosolic constituents from processes occuring inside. The third theme is that proteolysis and phosphorylation may be intimately connected with phosphorylation providing signals for protein activation/inactivation as well as degradation [21, 41, 125, 129, 153, 183, 198, 218]. In addition to degrading proteins in their entirety, proteolytic systems can also partially degrade polypeptides or can selectively remove individual subunits from oligomeric proteins complex. In some cases, these subunits function as inhibitors or activators thus providing another level of metabolic regulation. From an understanding of the mechanisms of protein breakdown, several biotechnological applications have emerged, especially the ability to manipulate protein half-lives *in vivo*

[100]. Clearly, as more is known of this fascinating process, its applications to agriculture and medicine could be profound.

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