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Regulation of Protein Degradation

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INTRODUCTION

The intracellular level of a protein is dependent on both its rate of synthesis and its rate of degradation. Thus, differential regulation of protein stability represents a potential mechanism for modulating gene expression. Increasing evidence suggests that protein degradation is indeed a regulatory mechanism in vivo. Dramatic differences in the in vivo stability of different proteins have been documented. Multiple proteins have been demonstrated to undergo selective proteolysis only at particular stages in the cell cycle, after certain environmental stimuli, or after specific metabolic or developmental changes. The degradation of many intracellular proteins is ATP dependent, which does not appear necessary based on the energetics of peptide bond hydrolysis but which does provide a potential mechanism for regulation. Proteinase activities are not constant; some increase or decrease and others appear de novo, resulting in changes in intracellular proteolysis.

Selective protein degradation serves a wide variety of roles during the plant life cycle. During seed germination, hydrolysis of seed storage proteins provides amino acids for protein synthesis in the growing seedling. Seed storage proteins, in turn, are synthesized in part from amino acids produced by the proteolysis of vegetative proteins. In annual plant species, this synthesis is accompanied by leaf senescence. Thus, proteolysis plays an important role in reallocating organic nitrogen in the plant. In nonsenescing cells, selective proteolysis serves to reduce the toxic effects of inactivated, denatured, unassembled, and abnormal proteins by degrading them. It also serves to regulate flux through metabolic pathways by regulating the levels of rate-limiting enzymes. Another important cellular function of proteolysis is to modulate the levels of receptors; degradation after signal perception represents a mechanism for signal desensitization. Although it is not directly demonstrated in plants, it is likely that proteolysis of proteins that regulate the activity of cell cycle kinases controls the plant cell cycle transitions as demonstrated for the yeast and animal cell cycles. Indeed, regulated proteolysis is probably directly or indirectly involved in most cellular processes.

We are only beginning to elucidate the machineries that degrade proteins and how the activities of those machineries are regulated. Complexities impeding progress include the diversity of intracellular locales in which proteolysis occurs, the diversity of proteolytic activities and pathways present in cells, and the relatively low level of proteinase activities. Our lack of understanding of how to preserve in vivo proteolytic activities in vitro also has interfered with the identification, purification, and analyses of proteinases. Although studies using heterologous proteins or synthetic peptide substrates will continue to provide valuable information, such studies must at some point utilize in vivo substrates if the physiological roles of proteinases are to be defined. Finally, no particular global feature of proteins can be correlated with in vivo stability, again indicating the diversity of mechanisms present.

Proteins may be subjected to a limited number of proteolytic cleavages either in the process of intracellular targeting to their final destination or in the maturation of the initial translation product to produce a biologically active mature molecule. This review does not discuss these events but rather focuses on what is known about the events that regulate, or are responsible for, the initial cleavages of a protein ultimately destined for hydrolysis to free amino acids. Our current understanding of the protein degradation process is that it occurs in stages, with the initial endoproteolytic cleavage(s) being more species specific and rate limiting than subsequent hydrolysis to free amino acids by less specific endo- and exopeptidases. For this reason, this review limits discussion to endoproteinases, which are referred to here simply as proteinases.

Recent advances in our understanding of the regulation of protein degradation have been made on two fronts: first, in the identification of proteinases, changes in whose activity and/or abundance could or do result in the degradation of previously stable protein(s); and second, in the identification of structural changes in proteins that increase their susceptibility to proteolysis. Advances in these two areas made after a previous review on plant proteolysis (Vierstra, 1993) are elaborated in the following discussions. Recently, multiple cases have been documented in which increases in the levels of mRNAs encoding proteinases occur in response to developmental or environmental changes. The physiological significance of such increases remains unknown; however, they indicate physiological responses that may be mediated by proteolysis or that result in accelerated proteolysis and are therefore mentioned here. Cell cycle progression in eukaryotes represents an exquisite example of the importance of regulated protein degradation, and recent data regarding proteolysis and cell cycle transitions are discussed. A brief description of the diversity of proteinases present in plants is presented, focusing on recent work on

Table 1. Classes of Endoproteinases

Class	Diagnostic Inhibitors[a]	Notable Characteristics
EC 3.4.21 serine proteinase	DFP, PMSF, TI, aprotinin	Contain conserved histidine residue; at least four evolutionarily distinct superfamilies
EC 3.4.22 cysteine proteinase	Iodoacetate, iodoacetamide	Both acid and neutral pH optima; thiols activate
EC 3.4.23 aspartic proteinase	Pepstatin	Acidic pH optima
EC 3.4.24 metalloproteinase	EDTA; 1,10-phenanthroline	Typically requires zinc
EC 3.4.99 not known	None available	

[a] DFP, diisopropylphosphorofluoridate; PMSF, phenylmethanesulfonyl fluoride; TI, trypsin inhibitor.

chloroplast proteinases. Space limitations prevent a complete discussion of all proteinases present. This review also discusses possible future research directions.

PROTEINASES IN PLANT CELLS

In contrast to the classification of most other enzymes, classification of endoproteinases is made on the basis of the active site residue, not the substrate (Barrett, 1986). Endoproteinases divide into four major groups: cysteine proteinases, serine proteinases, metalloproteinases, and aspartate proteinases. A fifth group consists of endoproteinases whose catalytic mechanism cannot be classified with any of the previous four (Table 1). An endoproteinase is classified into one of the four groups through the effect of active site inhibitors, the requirement for metal ions, and whether thiol compounds activate the enzyme (Table 1).

Although all four classes of proteinase have been described in plant cells, most described to date from vegetative organs are cysteine proteinases (for reviews, see Ryan and Walker-Simmons, 1981; Storey, 1986). Most of these cysteine proteinases have acidic pH optima in vitro, suggesting that they are localized to the vacuole in vivo. In support of this, vacuoles isolated from Melilotus mesophyll protoplasts contain the majority of proteinase activities present in total cellular extracts (Canut et al., 1987). Many specific cysteine proteinases have been localized to the vacuole. In addition, the other classes of proteinase have been localized to the vacuole in several species (Figure 1; Storey, 1986).

Distinct from these proteinases is a highly conserved 600- to 900-kD proteolytic complex present in the cytosol and nucleus of eukaryotic cells and in archaebacteria that has been variously referred to as the proteasome, 20S protease, macropain, or multicatalytic protease (for reviews, see Orlowski, 1990; Rivett, 1993). In contrast to the aforementioned proteinases, there are at least three, and probably more, distinct proteolytic activities present in the proteasome (Orlowski et al., 1993). The proteasome has a neutral pH optimum, distinguishing it from vacuolar proteinases. The cleavages performed by the proteasome are relatively nonspecific, and

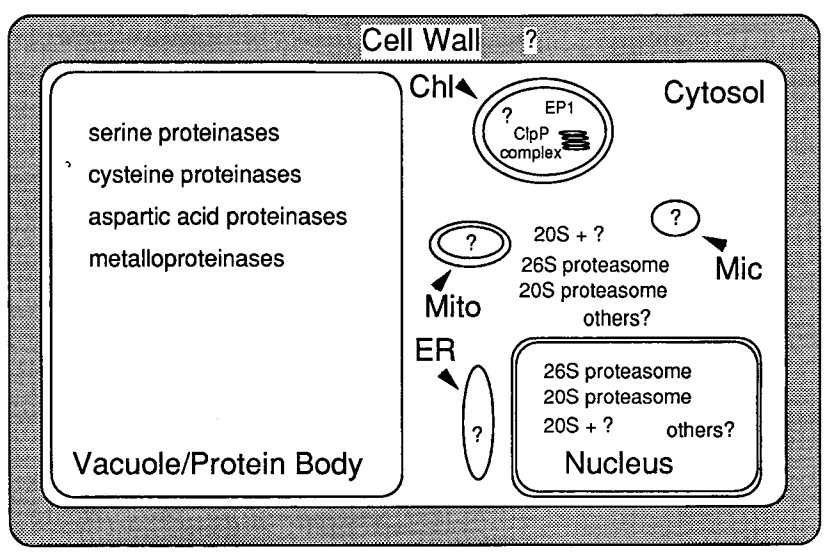

Figure 1. Intracellular Locations of Plant Endoproteinases.

Diagrammatic representation of the subcellular organelles and the proteinases that have been identified in the corresponding compartments. A question mark indicates that additional types of endoproteinolytic activities probably exist in the indicated organelle but have not yet been characterized. The specific endoproteinases listed are described in the text. The organelles are not drawn to scale. Chl, chloroplast; ER, endoplasmic reticulum; Mic, microbody; Mito, mitochondrion.

it typically produces fragments containing from six to nine amino acids (Wenzel et al., 1994). These properties are in marked contrast to the proteinases described previously, which have very limited cleavage site specificities and produce detectable protein products. Although several of the proteasome cleaving activities can be related to the four classes of proteinase through the use of class-specific inhibitors and peptide substrates, none of the proteasome subunits shares any amino acid sequence identity with any known proteinase. Recently, the N-terminal threonine residue on the β-subunit (see later discussion) of the archaebacterial enzyme has been identified as the active site residue, revealing that this enzyme has a novel type of proteolytic mechanism (Seemuller et al., 1995). In summary, in almost all its properties, the proteasome is characteristically different from the other classes of proteinases.

Electron microscopic studies have shown that all proteasomes characterized to date share a similar structure: they are hollow cylinders consisting of four stacked rings, each with sevenfold symmetry (Rivett, 1993). A crystal structure for the proteasome of the archaebacterium *Thermoplasma acidophilum* supports this model (Lowe et al., 1995). The archaebacterial enzyme consists of multiple copies of two different subunits, α and β (Zwickl et al., 1992). The yeast proteasome consists of 14 different polypeptides ranging from 22 to 35 kD in size, each of which resembles either the α or β subunit of the archaebacterial enzyme (Heinemeyer et al., 1994). The two outer rings of all proteasomes contain α or α-like subunits; the inner rings contain β or β-like subunits. The α-like subunits are postulated to provide some selectivity by covering the catalytic sites, allowing only unfolded proteins access to the central cavity with its active-site threonine residues (Lowe et al., 1995).

The higher plant proteasome has been characterized biochemically (Arrigo et al., 1987; Schliephacke et al., 1991; Yang and Malek, 1991; Ozaki et al., 1992). Genes for two α-like and one β-like subunit have been cloned from Arabidopsis (Genschik et al., 1992b, 1994; Shirley and Goodman, 1993). DNA sequence encoding one of the α-like subunits is contained within a region of the genome that can be deleted without apparent phenotypic consequences (Shirley and Goodman, 1993). This Arabidopsis subunit is most similar to the yeast α-like subunit PRE6, whose gene is essential (Heinemeyer et al., 1994). Whether there are redundancies in higher plant proteasome subunits not present in yeast awaits characterization of more subunits of the higher plant enzyme and determination of its composition.

In yeast, the proteasome has been implicated in the degradation of proteins containing amino acid analogs, of short-lived regulatory proteins, of unassembled subunits, and of cell cycle–regulated proteins (Hilt et al., 1993). Yeast cells with a point mutation in one β-like subunit are defective in the hydrolysis of multiple proteins and are sensitive to heat stress (Heinemeyer et al., 1991). The role of the proteasome in plant cells is not known, although it has been found that, as in all other eukaryotes, it is the catalytic component of a larger proteinase that participates in the ubiquitin-dependent pathway for proteolysis (see later discussion; for recent reviews, see Vierstra, 1993; Ciechanover and Schwartz, 1994). Clearly, the activity of the proteasome must be regulated to prevent premature or unwanted proteolysis of cytosolic and nuclear proteins.

Advances in our knowledge of proteinases in the chloroplast have also been made recently. A chloroplast gene homologous to the catalytic subunit of an ATP-dependent bacterial proteinase called ClpP has been identified (Maurizi et al., 1990), and its expression in chloroplasts has been verified by immunolocalization with antibodies raised against bacterially expressed tobacco ClpP (J. Shanklin, unpublished data). Bacterial ClpP is activated by one of several different homologous proteins such as ClpA and ClpB, which have ATPase activity. Plant nuclear genes encoding ClpA/ClpB–like polypeptides have been identified from several species, including tomato (Gottesman et al., 1990), pea (Moore and Keegstra, 1993), Brassica (Ko et al., 1994), and tobacco (J. Shanklin, unpublished data). Although the Brassica and pea cDNAs to ClpA-like proteins were isolated during screens for inner envelope proteins, the ClpA/ClpP complex appears to be solely stromally localized (J. Shanklin, unpublished data).

Another proteinase, EP1, has recently been isolated from pea chloroplasts. This metalloproteinase also appears to be stromally localized (Bushnell et al., 1993). In vitro, EP1 degrades the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit to a smaller polypeptide of 36 kD, suggesting that ribulose-1,5-bisphophate carboxylase/oxygenase is an in vivo substrate of this proteinase (Bushnell et al., 1993). How EP1 and the ClpP enzymes are regulated in the chloroplast stroma is not known; however, one area for future investigation is to determine whether, like the bacterial proteinase, the chloroplast ClpP/ClpA may be regulated by in vivo Mg^{2+} and/or ATP levels.

DEVELOPMENTAL AND PHYSIOLOGICAL REGULATION OF PROTEINASE ACTIVITY

Proteolysis can be regulated by controlling the abundance, localization, and/or activity of proteinases catalyzing the hydrolysis of the substrate proteins. Thus, both transcriptional control of proteinase gene expression and post-translational mechanisms for the activation or inhibition of existing proteinases have been demonstrated in plant experimental systems. Examples of developmental and metabolic changes accompanied by changes in proteolytic activity are detailed in the following sections.

Seed Storage Protein Degradation

Storage proteins synthesized during seed maturation (see Shewry et al., 1995, this issue) are degraded during germination to small peptides or amino acids that are subsequently transported to the growing seedling (Preston and Kruger, 1986; Wilson, 1986; Fincher, 1989). Typically, storage proteins are first cleaved by specific endoproteinases; the resulting peptides

are then hydrolyzed to free amino acids by the action of multiple, less specific exopeptidases and/or endopeptidases. Although it is possible to detect proteolytic activity in extracts from dry seeds, the proteinases responsible for the initial cleavages of the storage proteins of most species increase during germination due to de novo synthesis. However, in some species, proteinases active in storage protein hydrolysis are synthesized during seed maturation and remain inactive until germination.

To understand the regulation of storage protein proteolysis, it is necessary to correlate substrates with proteinases. This has proven difficult, in part because of the presence in germinating seeds of multiple activities capable of cleaving storage proteins in vitro. A specific endoproteinase is considered to be responsible for the degradation of a specific seed storage protein if, in addition to the ability to cleave the storage protein in vitro, one or both of the following criteria are met: it colocalizes with the substrate (which in this case often means colocalization in protein bodies), or the appearance of its activity correlates with the disappearance of a particular storage protein. Although these criteria are not entirely satisfactory for definitive assignment, they provide a first test.

The characterization of developmentally regulated seed storage proteinases is most complete for members of the cereal and legume families. During cereal germination, scutellar epithelia and aleurone layers that adjoin or surround the protein storage endosperm secrete a complex group of proteinases, which was first described in barley by Jacobsen and Varner (1967). These proteinases include two groups of cysteine proteinases: EP-A (Hammerton and Ho, 1986; Koehler and Ho, 1988, 1990a) and EP-B (Koehler and Ho, 1990a, 1990b). Two members of the EP-B family share 98% amino acid identity. Identification of the relationship between EP-A and EP-B isozymes awaits the isolation of sequences corresponding to EP-B. In vitro, both enzyme families degrade the B and D types of hordein, the abundant alcohol-soluble endosperm storage protein of barley seeds (see Shewry et al., 1995, this issue), suggesting that these hordeins are their in vivo substrates.

Additional proteinase activities from germinating barley endosperms have been identified using an in situ gel assay (Wrobel and Jones, 1992; Zhang and Jones, 1995). These additional proteinases include members of the other classes of proteinases. Multiple germination-induced proteinase activities have also been described in rice (Watanabe et al., 1991) and maize (de Barros and Larkins, 1990; Mitsuhashi and Oaks, 1994). Different proteolytic activities in maize are hypothesized to be responsible for the degradation of specific zeins based on the time course of proteinase activity appearance and zein disappearance (de Barros and Larkins, 1990; Mitsuhashi and Oaks, 1994).

In legumes, recent efforts have been directed toward purifying the proteinase activities responsible for germination-specific degradation of cotyledon storage proteins (Wilson, 1986; Wilson et al., 1988). Purified proteinase C1, which catalyzes cleavage of the 66-kD α and the 76-kD α′ subunits of β-conglycinin in germinating soybean cotyledons, is only weakly active against the other β-conglycinin subunit and against another major storage protein, glycinin, suggesting that additional proteinases degrade these proteins (Qi et al., 1992). The site of proteinase C1 cleavages in the β-conglycinin α and α′ subunits has been mapped to the N-terminal one-third of both, such that 48- and 58-kD products are generated from the α and α′ subunits, respectively (Qi et al., 1994). Whereas the majority of the proteinase activities in germinating barley seeds are cysteine proteinases, soybean C1 appears to be not a cysteine proteinase but a serine proteinase with an acidic pH optimum (Qi et al., 1992).

A different proteinase is thought to be responsible for the initial cleavages of two other soybean storage proteins, Kunitz and Bowman-Birk trypsin inhibitors (Papastoitsis and Wilson, 1991). Its dependence on a thiol for activity in vitro and its inhibitor sensitivity profile classify this enzyme as a cysteine proteinase. In contrast, the proteinase responsible for the initial degradation of the mung bean Bowman-Birk trypsin inhibitor appears to be a serine proteinase. This mung bean activity is also distinct from the soybean proteinase in that it is present in the protein bodies of dry seed rather than appearing de novo during germination (Wilson and Tan-Wilson, 1989).

A cDNA encoding a putative cysteine proteinase has recently been isolated from germinating chick pea (*Cicer arietinum*). mRNA hybridizing to this cDNA is not present in dry seed but appears within 24 hr after imbibition. mRNA levels are also regulated by ethylene (Cervantes et al., 1994). The in vivo substrate of this proteinase has not been identified.

For those proteinases present in the dry seed, work in buckwheat and rice has identified a possible mechanism regulating their activity. A metalloproteinase capable of cleaving the major storage protein of buckwheat was purified from the dry seed (Belozersky et al., 1990). Although this proteinase is colocalized with its substrate in protein bodies when synthesized during seed maturation, premature proteolysis of the substrate may be prevented by an inhibitor of the proteinase, whose activity is highest in the dry seed (Elpidina et al., 1991). Premature proteolysis by this metalloproteinase may also be regulated by the availability of metal ions essential for its activity (Elpidina et al., 1991). In rice, a cysteine proteinase inhibitor is expressed during seed maturation, and the purified inhibitor is effective in blocking the activity in vitro of a rice proteinase (Kondo et al., 1990). This suggests that this inhibitor may modulate proteinase activity in vivo.

Thus, multiple mechanisms modulate proteinase activity during seed germination. Both transcriptional regulation and post-translational mechanisms ensure that proteinases are present and active at the appropriate time.

Environmental Stress Regulates Proteinase Gene Expression

Several mRNAs that encode proteins with amino acid identity to known proteinases, mostly cysteine proteinases, have been

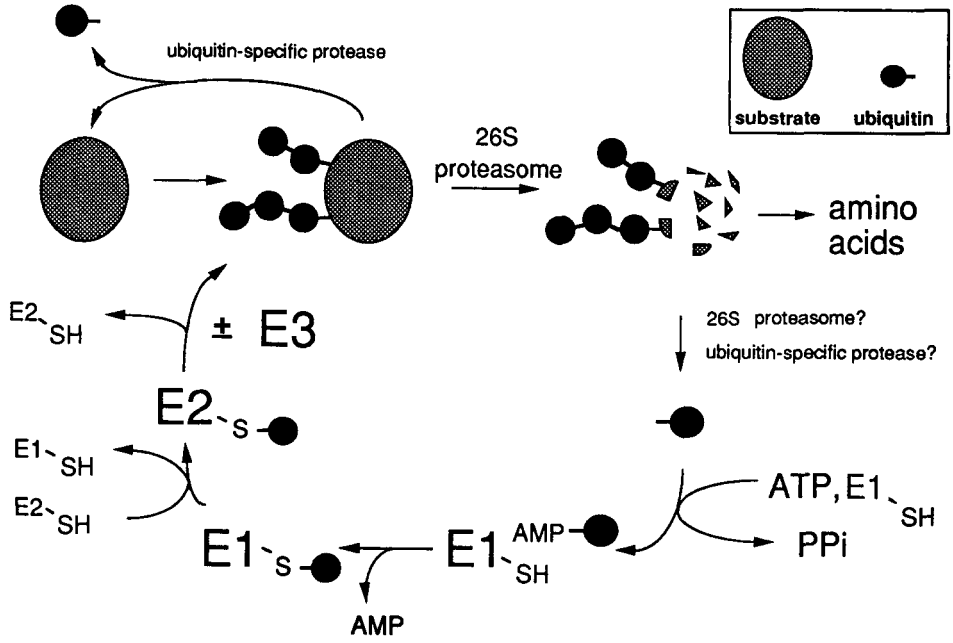

Figure 2. Diagrammatic Representation of the Ubiquitin-Dependent Proteolytic Pathway.

The enzymes required for the covalent attachment of the protein ubiquitin and for the catabolism of ubiquitinated proteins are indicated. E1, ubiquitin-activating enzyme, binds ubiquitin, forms a ubiquitin adenylate, and catalyzes the formation of a thioester-linked ubiquitin to one of its cysteine residues. E1 then transfers activated ubiquitin to one of a family of proteins called E2, or ubiquitin-conjugating enzymes. E2s then transfer activated ubiquitin to a lysyl residue of substrate proteins, with some transfers requiring a third component, E3 (ubiquitin ligase). Ubiquitin-specific proteases hydrolyze the ubiquitin–protein and/or ubiquitin–ubiquitin linkages, releasing both components intact. Related enzymes, possibly as part of the 26S proteasome itself, also cleave off peptides remaining attached to ubiquitin after 26S proteasome action on ubiquitinated proteins, allowing ubiquitin to be reused.

shown to accumulate when tissues are exposed to different environmental stresses. mRNAs encoding for two different cysteine proteinases accumulate in drought- or salt-stressed Arabidopsis plants but not in plants treated with abscisic acid (Koizumi et al., 1993). In contrast, mRNA encoding a cysteine proteinase induced in drought-stressed pea is also induced after incubation with abscisic acid (Guerrero et al., 1990). Levels of a mRNA encoding a cysteine proteinase increase in tomato fruit after cold treatment (Schaffer and Fischer, 1988). However, little is understood concerning the physiological role of these changes in proteinase mRNA levels. Indeed, it remains to be seen whether corresponding changes occur in proteinase protein levels. In addition, intracellular locations and in vivo substrates need to be identified.

High-temperature treatment of eukaryotic and prokaryotic cells (heat shock) increases their capacity to degrade proteins. This results from the induction of synthesis of proteinases, such as proteinase La in *Escherichia coli*, or the induction of ubiquitin and components of the ubiquitin-dependent pathway for proteolysis in eukaryotes (Figure 2; Goldberg, 1992). This pathway requires the covalent attachment of the 76-amino acid protein ubiquitin to substrate proteins. Some ubiquitin transcripts increase after heat shock treatment in plants (Burke et al., 1988; Christensen et al., 1992; Garbino et al., 1992; Genschik et al., 1992a), as has been previously demonstrated for animal and yeast ubiquitin genes (Finley and Chau, 1991). Components of the ubiquitin pathway are essential for viability at elevated temperature in yeast (Seufert and Jentsch, 1990). The heat induction of other proteinases has not yet been demonstrated in eukaryotes, suggesting that only the ubiquitin-dependent pathway may be required under these conditions. Other proteins whose synthesis is induced under heat-shock conditions include members of chaperonin families. These proteins have been shown to bind to a variety of intracellular proteins, either to prevent aggregation or to facilitate degradation (Goldberg, 1992). The relationship between chaperonin binding and proteolysis is a fertile area for future research.

Cell Death/Senescence Includes Changes in Proteinase Gene Expression

The process of cellular death includes degradation of resident macromolecules, including proteins. Protein breakdown can be achieved by preexisting cytosolic, vacuolar, or cell wall proteinases or by newly synthesized proteinases that localize to

one or all of the aforementioned compartments. One model system recently developed to study programmed cell death in plants is the in vitro differentiation of *Zinnia elegans* mesophyll cells into xylem elements (for review, see Church, 1993). This process involves autolysis of the cellular contents and deposition of lignin in the cell wall. The isolation of a partial cDNA encoding a cysteine proteinase whose mRNA is induced coincident with secondary wall thickening suggests that changes in proteinase gene expression accompany this differentiation (Ye and Varner, 1993). A full-length cDNA for this cysteine proteinase contains a putative signal peptide, indicating transport into the endomembrane system (Z.-H. Ye and J. Varner, unpublished data). Tissue prints demonstrate that the protein is intracellular, suggesting that the specific intracellular locale may be the vacuole. Although mRNA levels for this cysteine proteinase are elevated during in vitro xylogenesis, transcripts can also be detected at lower levels in leaves, stems, roots, and flower buds. Proteinase mRNA can only be detected in developing tracheary elements in tissue prints of stem sections and not in mature xylem elements or phloem, consistent with its hypothesized role in the cell death process in vivo (Z.-H. Ye and J. Varner, unpublished data). The identification of additional proteolytic activities induced during tracheary element differentiation suggests that a battery of proteinases may be responsible for autolysis (E. Beers, unpublished data). Elucidation of the intracellular location, kinetics of accumulation, and substrate specificities of these newly described proteinase activities will delineate their roles in this example of programmed cell death.

Another example of a plant cell death process in which expression of proteinases is induced is senescence. Two cDNAs encoding amino acid sequences related to cysteine proteinases have been isolated from senescing Arabidopsis leaves (Hensel et al., 1993; Lohman et al., 1994). Transcript levels of one, SAG12, are maximally 100-fold higher in senescent leaves with 50% chlorophyll loss than in mature leaves with no visible chlorophyll loss (Lohman et al., 1994). mRNA for another, SAG2, is present at two- to fourfold higher levels (on a per leaf basis) in senescent leaves than in mature green leaves (Hensel et al., 1993).

A distinct senescent process under study is the degeneration of unpollinated pea ovaries, ultimately resulting in abscission of these organs. If pollination has not occurred by 3 days post-anthesis, physiological and metabolic changes occur in the ovule, including increases in proteinase activities and cell lysis (Vercher et al., 1989). A cDNA for a cysteine proteinase induced during this process has been isolated (Granell et al., 1992). The corresponding mRNA is also present at lower levels in other organs. The open reading frame encoded by the cDNA contains an N-terminal sequence with a hydropathy plot consistent with signal peptides found on other proteinases, although its cellular location is not known. In situ hybridization localizes mRNA for this protein in the funiculus, integuments, and vascular bundles of the ovule as well as in the ovary endocarp (Granell et al., 1992). The amino acid sequence of this protein most resembles a gibberellin-induced proteinase from rice seeds (Watanabe et al., 1991).

Possibly analogous to the physiological changes that occur during cell death are the changes that occur during wounding. A tobacco mRNA encoding a proteinase with amino acid identity to cysteine proteinases accumulates sixfold after incision wounding. This accumulation is slow, taking 48 hr (Linthorst et al., 1993). mRNA levels corresponding to this proteinase also fluctuate in tobacco leaves in a circadian rhythm. The physiological relevance of this modulation of mRNA levels is unknown. It is apparent that modulation of proteinase gene expression is occurring during plant senescent processes; future work should identify the cellular role for these enzymes and the mechanism of their induction.

Regulation of Proteasome Activity

Few data are available concerning regulation of the activity and/or specificity of the plant proteasome, the major cytosolic and nuclear proteinase complex (Figure 1). However, in animal cells, three different types of modifications to the proteasome have been well documented: alteration of subunit composition by synthesis and incorporation of new subunits, post-translational modification of preexisting subunits, and binding of additional protein complexes. Binding of one complex to the plant proteasome has been demonstrated (see later discussion). The high degree of structural and amino acid sequence conservation among all proteasomes suggests that the other modifications may occur in plant cells as well.

The subunit composition of the proteasome in mammalian cells changes in response to γ-interferon exposure, altering the nature of the peptides produced by the enzyme (Goldberg and Rock, 1992; Driscoll, 1994). There is evidence for modification of proteasome subunits by limited proteolysis, glycosylation, or phosphorylation in several different animal species (Rivett, 1993). The relationship between post-translational modifications and the regulation of enzyme activity is not known; indeed, whether the limited proteolysis seen represents in vivo or in vitro events has not yet been determined. However, both changes in subunit composition and subunit modification represent possible mechanisms by which the in vivo activity of this enzyme could be modulated.

The binding of modulator proteins or complexes of proteins represents an important mechanism to modulate proteasome activity (Goldberg, 1992; Rechsteiner et al., 1993). Multiple protein complexes that interact with the mammalian proteasome have been identified and are classified as either activators, inhibitors, or modulators of the former two types (DeMartino and Slaughter, 1993). Upon binding to the proteasome, one specific complex inactivates the enzyme toward some protein and synthetic peptide substrates but activates it toward proteins that have been modified by the covalent addition of ubiquitin. This modified proteasome has been given a separate designation, the 26S protease or 26S proteasome. ATP

is required both for assembly of the modulator complex to the 20S core and for activity of the 26S proteasome after assembly (Ganoth et al., 1988). The importance of the 26S proteasome in cellular physiology has been revealed by multiple studies. For example, mutations in subunits of the 26S modulator complex block cell cycle progression in both fission and budding yeasts in mitosis (Ghislain et al., 1993; Gordon et al., 1993), implicating the 26S proteasome in cell cycle progression.

The 26S proteasome, originally identified in plant extracts as an ATP-dependent activity capable of degrading ubiquitinated proteins, has been purified from spinach leaves (Fujinami et al., 1994). Two distinct forms have been identified by gel electrophoresis, and both are active against peptide substrates. The biological significance of these two forms is unknown. No other additional complexes that bind to the proteasome have been identified in plant extracts; however, it is likely that these complexes exist in plants as they do in animals.

Regulation of Activity of Other Proteinases

Ca^{2+}-dependent cysteine proteinases have been described in animals and fungi and include the well-characterized calpains of vertebrates (Mellgren and Murachi, 1990). The demonstration of a Ca^{2+}-stimulated proteinase activity in Arabidopsis root extracts suggests that modulation of intracellular Ca^{2+} levels could affect the activity of one or more proteinases in vivo (Reddy et al., 1994).

CHANGES IN PROTEINASE SUBSTRATES RENDER THEM MORE SUSCEPTIBLE TO PROTEOLYSIS

Proteins undergo a variety of post-translational modifications. Much work has been directed toward identifying whether post-translational changes regulate in vivo stability, and, if so, which ones (Stadtman, 1990). The most notable modifications linked to protein stability include oxidation of amino acid residues, phosphorylation, acetylation, and ubiquitination. Ubiquitination has been confirmed as a signal for degradation via the 26S proteasome (Ciechanover and Schwartz, 1994). Attachment of ubiquitin requires two to three enzymes (Figure 2): a ubiquitin-activating enzyme (E1); a family of ubiquitin-conjugating enzymes (E2s or UBCs); and, sometimes, a ubiquitin ligase (E3). E1 and several E2 proteins have been biochemically characterized and cloned from wheat germ (Vierstra, 1993). To date, 15 different E2 proteins have been cloned from Arabidopsis (Bartling et al., 1993; Girod et al., 1993; Sullivan et al., 1994).

Specificity of ubiquitin attachment is determined in part by the nature and activity of E2s present. However, the substrate may have to be modified to be ubiquitinated. For example, the yeast transcription factor Gcn4 (Kornitzer et al., 1994) and the yeast cyclins Cln2 (Deshaies et al., 1995) and Cln3 (Yaglom et al., 1995) require phosphorylation prior to ubiquitination. In contrast, the ubiquitin-dependent degradation of the Xenopus proto-oncogene protein Mos requires the dephosphorylated form of Mos (Nishizawa et al., 1993). These data suggest that multiple levels of post-translational modification regulate degradation of a single species. Thus, studies on the nature of post-translational modifications to proteins and the regulation of their attachment may provide insights into how intracellular proteolysis is regulated. Recent advances in our understanding of the relationship between post-translational modifications and the regulated degradation of selected plant proteins are detailed in a later section.

Phytochrome A Is Degraded upon Light Absorption

Protein degradation, in addition to transcriptional regulation, plays an important role in determining the level of the photoreceptor phytochrome in developing seedlings upon exposure to light. In dark-grown oat seedlings, the most abundant phytochrome isoform is phytochrome A (PhyA), which is present in the red light–absorbing P_R form (Quail, 1991). The half-life of P_R has been determined to be ~100 hr. Upon red light absorption, P_R is converted into the biologically active far-red-light–absorbing P_{FR} form, which exhibits a half-life of ~1 hr (for review, see Vierstra, 1994). This light-mediated down-regulation of PhyA stability is consistent with the observed degradation of other receptors after signal perception.

Correlated with the change in PhyA protein stability in vivo are structural changes in the molecule (Lagarias and Mercurio, 1985), energy-dependent aggregation (Quail and Briggs, 1978), and ubiquitination (Shanklin et al., 1987). Ubiquitinated PhyA is found preferentially in aggregates early after light absorption (Jabben et al., 1989). PhyA in aggregates is unstable, whereas the soluble PhyA pool appears to be stable. Based on the differential reaction of denatured ubiquitinated PhyA and unmodified PhyA to phytochrome monoclonal antibodies, one site of possible ubiquitin attachment or altered accessibility due to ubiquitination has been identified (Shanklin et al., 1989; Vierstra, 1994). This site maps to form-dependent proteinase cleavage sites, suggesting a relationship between structural differences and ubiquitination (Shanklin et al., 1989).

Other factors that may modulate PhyA stability in vivo include post-translational modifications to the protein. For instance, one known in vivo post-translational modification is the phosphorylation of N-terminal serine residues (McMichael, 1991). PhyA is recognized by the ubiquitin-dependent conjugation pathway, and the role structural changes in the molecule play in the recognition process needs to be determined.

Rate-Limiting Enzymes Are Potential Targets for Specific Proteolysis

An important cellular function for selective proteolysis is to regulate metabolic flux by regulating the levels of rate-limiting

enzymes in the pathway. One well-studied example of an enzyme thought to regulate flux is nitrate reductase (NR). NR catalyzes the reduction of nitrate to nitrite, which is considered to be the rate-limiting step in the nitrogen assimilatory pathway. Although regulation of NR mRNA, protein levels, and enzyme activity is complex and depends on the organ and environmental conditions (see Crawford, 1995, this issue), in general NR protein increases in the presence of nitrate and declines when nitrate is removed. In both roots and shoots from 6-day-old maize seedlings, removal of nitrate from the medium results in rapid inactivation of NR activity, which is followed 2 to 4 hr later by loss of NR protein (Li and Oaks, 1993). NR in maize shoots is also sensitive to light; incubation in the dark mimics the response seen upon nitrate removal, that is, rapid loss of activity with a delayed loss of protein (Li and Oaks, 1994). Dark-mediated inactivation in maize shoots is reversible after a short period.

A possible mechanism for inactivation comes from work on spinach leaf NR, which is modified by phosphorylation, which inactivates the protein (for review, see Huber et al., 1993; Kaiser and Huber, 1994). NRs from other species may be similarly modified (LaBrie and Crawford, 1994). In addition, a 100-kD protein that binds to inactive NR has been identified (Spill and Kaiser, 1994; MacKintosh et al., 1995). One hypothesis is that phosphorylation provides a signal for degradation by proteinases. A maize root serine proteinase that cleaves NR in vitro has been characterized, but whether it initiates the proteolysis of NR in vivo remains to be determined (Goodfellow et al., 1993).

The enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which catalyzes the conversion of *S*-adenosyl-L-methionine to ACC, is considered to be the rate-limiting step in the biosynthesis of the plant growth regulator ethylene. ACC synthases have relatively short half-lives in vivo, from 20 min to 2 hr, depending on the organ and species (Kende, 1993). Virtually nothing is known about the machinery responsible for degrading ACC synthase. Degradation of the enzyme is slowed in wounded tomato fruit disks after incubation of the disks with uncouplers of oxidative phosphorylation, suggesting a requirement for ATP (Kim and Yang, 1992).

There are two described modifications of ACC synthase. ACC synthase is inactivated in vitro by covalent linkage of the aminobutyrate portion of its substrate, *S*-adenosyl-L-methionine (Yip et al., 1990). Evidence suggests that this mechanism may operate in vivo (Satoh and Yang, 1989), although contradictory evidence has also been reported (Spanu et al., 1990). In elicitor-treated tomato suspension cells, inhibitors of protein kinases rapidly reverse the activation of ACC synthase and increase the rate of inactivation of the enzyme (Spanu et al., 1994). Phosphatase inhibitors enhance the elicitor-induced increase in activity, suggesting that the activity of ACC synthase is linked to protein phosphorylation events. Although the targets of these phosphorylation events are not known, these results are intriguing and lead to questions about whether phosphorylation events might mediate degradation of this protein as well as its inactivation.

Unassembled Proteins Are Degraded Rapidly

It is well established that protein subunits or apoproteins typically do not accumulate in cells when unable to assemble with their cofactors or other protein subunits. These unassembled subunits appear to be degraded selectively. In plants, this phenomenon has been demonstrated for plastid complexes composed of nuclear- and chloroplast-encoded subunits as well as for complexes that require assembly with cofactors such as heme, metal ions, or chlorophyll (Schmidt and Mishkind, 1983; see von Wettstein et al., 1995, this issue). We know little about the way the cell recognizes unassembled proteins and the machinery that degrades them. One example of this phenomenon is the synthesis of the electron transfer protein plastocyanin in *Chlamydomonas reinhardtii.* The protein, apoplastocyanin, requires a copper cofactor and does not accumulate in cells grown without it (Merchant and Bogorad, 1986). Apoplastocyanin is synthesized at the same rate in copper-sufficient and copper-deficient cells; however, the apoprotein is rapidly degraded under the latter growth conditions (S. Merchant, unpublished data). Apoplastocyanin is more susceptible to proteinases in vitro than is the holoenzyme. Comparison of their secondary structures indicates that the two forms may not have the same conformation in solution in vitro, suggesting that the apoprotein may be distinguished from the holoprotein in vivo. However, in vivo instability also requires a proteinase activity that may be present only under copper-deficient growth conditions (S. Merchant, unpublished data). Therefore, changes in proteolytic activity and structural changes in apoplastocyanin itself may regulate the intracellular levels of apoplastocyanin in the absence of copper.

Stoichiometry of cytosolic complexes is also maintained in part by protein degradation. The α subunit of the yeast fatty acid synthetase complex has been shown to be rapidly degraded when not assembled with its other subunit (Egner et al., 1994). The proteasome is implicated in degradation of the α subunit because the protein is stabilized in a strain carrying a point mutation in a proteasome subunit (Egner et al., 1994). The stability of yeast ribosomal proteins also appears to be a function of their assembly into the ribosome; unassembled subunits are degraded rapidly (Woolford and Warner, 1991).

REGULATION OF PROTEOLYSIS IN CELL CYCLE PROGRESSION

Virtually nothing is known about proteolytic events regulating the cell cycle in higher plants. However, because both the process of cell division and the components of cell cycle regulation are highly conserved, it is likely that events similar to those elucidated in yeast and mammalian cells regulate the higher plant cell cycle. Elegant studies have demonstrated that regulated proteolysis plays critical roles at specific checkpoints in the cell cycle. Work in budding and fission yeasts and in

metazoans has shown that proteolysis of cyclins and cyclin kinase inhibitors is essential for exit from mitosis (Glotzer et al., 1991; Seufert et al., 1995) and for initiation of DNA replication (the G1/S transition; Schwob et al., 1994).

Although the ubiquitin-dependent proteolytic machinery is known to be responsible for these degradation events, the process by which the substrates are recognized by the ubiquitin-conjugating enzyme E2 remains to be elucidated. It is probable that a combination of changes in the proteolytic machinery (presence/absence/activation of specific ubiquitin-conjugating enzymes) and a modification of the substrate (for example, phosphorylation) contribute to the observed specificity. The E2 enzyme encoded by the *UBC3* gene, which is required for the G1/S transition in yeast, is phosphorylated in vivo (Goebl et al., 1994). A potential substrate of UBC3, p40, is phosphorylated in a cell cycle–dependent manner (Schwob et al., 1994). In clam oocyte extracts, an additional activity required for cyclin B ubiquitination occurs only in mitotic extracts but can be stimulated in interphase extracts by Cdc2 kinase (Hershko et al., 1994). Additional substrates whose degradation is required for sister chromatid separation remain to be identified (Holloway et al., 1993). Thus, because of the conservation of the cell cycle, the rapid progress in understanding how proteolysis regulates cell cycle transitions in species from other kingdoms should reveal potential targets for proteolysis in higher plants.

PERSPECTIVES

Multiple Pathways May Regulate the Degradation of a Single Protein

Another feature of proteolysis is the use of multiple pathways to effect the degradation of a single species. This has been most thoroughly studied for the budding yeast α2 mating type transcriptional repressor. When fused to the N terminus of β-galactosidase, two domains of α2 target the fusion protein for rapid degradation (Hochstrasser and Varshavsky, 1990). Genetic studies indicate that these two domains operate via distinct mechanisms. One domain, Deg1, consists of the first 67 amino acids and targets the protein for degradation via the ubiquitin-dependent pathway, whereas the other domain, Deg2, consists of the C-terminal 75 amino acids and appears to be independent of the ubiquitin-mediated pathway (Chen et al., 1993). Mutations that result in nearly complete stabilization of a Deg1–β-galactosidase fusion protein only partially stabilize intact α2, again suggesting that this domain represents only one of the signals that participate in targeting this protein for rapid proteolysis.

There is also evidence that multiple pathways participate in the degradation of the yeast B-type cyclin, Clb2. The partial, rather than complete, stabilization of Clb2 in a yeast strain deleted for one of the ubiquitin-conjugating enzymes, UBC9, suggests that this cyclin may be degraded by multiple pathways, only one of which is mediated by UBC9 (Seufert et al., 1995).

Future Work

Recently, there has been a greater consideration of the role of proteolysis in controlling protein levels. Our knowledge of the particular proteolytic machinery responsible for the degradation of specific proteins and of the recognition mechanisms between the machinery and its substrate lags far behind our understanding of transcriptional regulation in plants, as it does in all other organisms. Future work aimed at identifying additional components of the ubiquitin-dependent proteolytic pathway and its physiological substrates will add greatly to our understanding of the role of this pathway in plant cells. In addition, identification of additional proteinases, their in vivo substrates, their subcellular locations, and the regulation of their levels and activities is necessary.

The role of plant vacuolar proteinases in the degradation of proteins in nonsenescing tissues remains to be clarified. Recent evidence implicates the yeast vacuole in the degradation of cytosolic protein by autophagy and digestion by resident proteinases (Takeshige et al., 1992; Egner et al., 1994). The presence of proteinases in higher plant vacuoles (Boller and Kende, 1979) and their activity toward intracellular proteins invite speculation that a similar mechanism may function in vascular plants. However, there is no direct evidence for such a mechanism in higher plants.

Another important area for future investigation is the role of post-translational modifications to specific substrates and proteinases in regulating proteolysis. A greater understanding of the nature of the interactions between substrate and proteinase and the role of post-translation modifications in these interactions should allow us to determine whether such modifications affect in vivo stability and activity, respectively. Information obtained from study of the regulation of proteolysis in other organisms will very likely add to our understanding of proteolysis in higher plants because there may be highly conserved mechanisms for altering protein stability. This would not be surprising, given the conservation of transcriptional and signal transduction mechanisms among eukaryotes.

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