Proteases and cellular regulation in plants
Mark Estelle

Protein degradation is accomplished by a diverse collection of proteases. Recent studies have illustrated the importance of proteolysis in the control of many aspects of cellular regulation from photosynthesis to photomorphogenesis. In addition, new results point to a role for proteolysis in programmed cell death, circadian rhythm, and defense response in plants.

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Abbreviations
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Aux	Auxin
AXR3	AUXIN RESISTANT3
COP1	CONSTITUTIVELY PHOTOMORPHOGENIC 1
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-protein ligase
EIR1	ETHYLENE INSENSITIVE ROOT1
FKF1	*Flavin-binding, Kelch repeat, F box1*
HY5	HYPOCOTYL5
IAA	INDOLE ACETIC ACID
PARP	poly(ADP-ribose) polymerase
PCD	programmed cell death
PSII	photosystem II
SCF	Skp1-cullin-F-box
SUMO	Small Ubiquitin-Related Modifier
TIR1	TRANSPORT INHIBITOR RESPONSE1
VAR2	*YELLOW VARIEGATED*
ZTL	*ZEITLUPE*

Introduction
The life span of most cellular proteins is significantly shorter than the life span of the organism. It follows therefore, that most proteins are degraded by cellular proteases of one sort or another. Some proteins are degraded when they wear out or become damaged. Other proteins are degraded when their constituents, carbon and nitrogen, are required to support the life of the organism. Still others are degraded in response to specific environmental or cellular signals. In each case, proteolysis is a specific and highly regulated process. This brief review highlights some of the most exciting research into proteolysis and cellular regulation in plants published during the past year. The studies described here illustrate the great diversity of cellular processes that depend upon regulated protein degradation, including photoinhibition in the chloroplast, programmed cell death, and photomorphogenesis in the developing seedling.

Proteases in the chloroplast
Protein degradation in chloroplasts, particularly in the photosynthetic apparatus, has been an important area of investigation for many years (reviewed in [1]). Not surpris-ingly given the endosymbiotic origin of the chloroplast, each of the chloroplast proteases described to date is related to a bacterial enzyme. During the past year, several interesting studies dealing with the function of FtsH-related proteases have been published. The *Escherichia coli* FtsH protein is an ATP-dependent metalloprotease and chaperone. It belongs to a larger family of proteins called the AAA proteins (ATPases associated with diverse cellular activities). Chloroplast FtsH-related proteins have been identified in pepper and *Arabidopsis* and shown to be localized to the stromal side of the thylakoid membrane ([2,3]; Figure 1). The *Arabidopsis* genome contains a number of *FtsH*-related genes. Two of these, *FtsH1* and *YELLOW VARIEGATED* (*VAR2*), have been characterized and appear to have distinct functions. The FtsH1 protein is involved in the degradation of the photosystem II (PSII) reaction-cen-

Figure 1
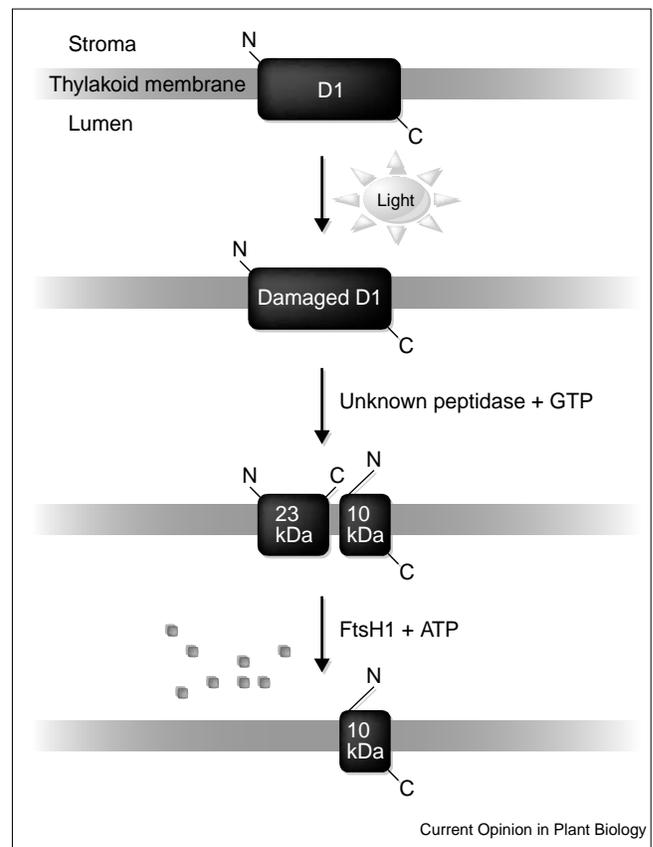
Model for degradation of the D1 protein after photooxidation. After photodamage, the protein is first cleaved into 10- and 23-kDa fragments by an unknown GTP-dependent protease. N and C represent the amino and carboxyl termini of the protein, respectively. According to the model proposed by Lindahl *et al.* [5••], the new carboxyl terminus generated by this event serves as a recognition motif for FtsH1, resulting in degradation of the fragment.

ter protein D1. When plants are exposed to intense light, reactive oxygen species are formed that cause irreversible damage to the D1 protein, thus arresting electron transport. This phenomenon is called photoinhibition. To recover from photoinhibition, the D1 protein must be removed from the reaction center and degraded. D1 is first cleaved into 10- and 23-kDa fragments by an unknown protease that is stimulated by GTP ([4]; Figure 1; see also Update). Recent results indicate that the 23-kDa fragment is then degraded by FtsH1 [5••]. As both D1 and FtsH1 are present in the membrane prior to photoinhibition, it is interesting to consider how the protease recognizes the damaged protein. One possibility, suggested by Lindhal *et al.* [5••], is that FtsH1 recognizes the new carboxyl terminus generated by the initial cleavage of damaged D1. By analogy with FtsH from *E. coli*, FtsH1 may also have chaperone activity that is required to extract damaged D1 from the reaction center concomitant with degradation.

The *VAR2* gene in *Arabidopsis* encodes a FtsH-related protein with 43% identity to FtsH1 [6••]. The two proteins are similar within the AAA domain but highly diverged in the amino- and carboxy-terminal regions. Mutations in *VAR2* result in an interesting variegated phenotype. Ultrastructural analyses of chloroplasts in white tissues indicate that VAR2 participates in chloroplast biogenesis. In the strong *var2-1* allele, however, there is no detectable mutant protein in either white or green tissues, suggesting that VAR2 is not essential for chloroplast biogenesis. Chen *et al.* [6••] suggest that there may be an activity that is able to compensate for the lack of VAR2 in some tissues. Why this putative activity compensates for the loss of VAR2 in some tissues but not in others, is an interesting question that awaits further investigation.

During its synthesis, the D1-reaction-center protein is the substrate of another protease. D1 is encoded by the chloroplast genome and is synthesized with a short terminal extension that must be cleaved after insertion of D1 into the thylakoid membrane. The cleavage is accomplished by a serine protease called CtpA. The X-ray structure of CtpA has now been solved at 1.8 Å resolution [7]. This structure will provide an important tool for further studies of the protease. Curiously, removal of the carboxy-terminal extension from D1 is required for the proper assembly of the Mn-cluster in PSII, but the absence of this extension in D1-truncation mutants of *Chlamydomonas* does not affect PSII function [8]. In the cyanobacterium *Synechocystis*, loss of the extension results in a decrease in the long-term fitness of the mutant strain [9]. Nevertheless, the physiological function of the extension is unclear.

ClpXP is another ATP-dependent protease in *E. coli*. This enzyme consists of a chaperone or regulatory subunit called ClpX and a protease subunit called ClpP. Both algal and higher plant chloroplast genomes encode a ClpP-related protein and nuclear-encoded chloroplast-targeted members of the ClpX family. The exact subunit composition of any specific ClpXP dimer has yet to be determined, but it is assumed that, in plants as in *E. coli*, the two subunits work together. To identify substrates of ClpP in *Chlamydomonas*, Majeran *et al.* [10••] reduced the levels of ClpP to 25–40% of those in the wild type by mutating the initiating codon to AUU. This genetic trick was necessary because cells completely lacking ClpP could not be recovered, presumably because it is an essential enzyme. The ClpP-deficient cells were still viable but accumulated higher than normal levels of the cytochrome b_6f complex when starved of nitrogen, conditions under which this complex is usually degraded. These results suggest that ClpP is responsible for the regulated degradation of the cytochrome b_6f complex.

Proteases and programmed cell death

In recent years, one of the best-studied examples of cellular regulation by proteases is that occurring during programmed cell death (PCD) in animal cells. The molecular era of PCD research began when genetic studies in *Caenorhabditis elegans* showed that a protease called CED-3 (Cell Death Protein-3) is required for PCD in this organism. CED-3 is similar to the interleukin-1-converting enzyme (ICE) from mammalian cells. Because these proteases have a cysteine in their active sites, and cleave at specific aspartic-acid residues, they were named caspases [11]. Since this finding, many new caspases have been identified in animal systems and implicated in PCD. Members of the caspase family are synthesized as inactive procaspases and cleavage-activated by a variety of external and internal signals. Their substrates include other procaspases, giving rise to an amplifying proteolytic cascade, as well as a variety of cellular proteins. PCD is the ultimate result of caspase activation.

Plants exhibit PCD in a number of contexts, most notably during the hypersensitive response to pathogen attack, tracheary-element differentiation, and senescence [12]. At present, it is not known if these instances of PCD involve a regulatory protease cascade. A standard BLAST search fails to detect an obvious caspase homolog in the recently completed *Arabidopsis* genome. A more careful examination of the database, however, revealed the existence of a distantly related family of proteins called the metacaspases [13••]. These proteins contain a caspase-like domain that includes the active-site cysteine and histidine. Intriguingly, some members of this family contain a zing-finger motif like that found in the *Arabidopsis* LSD1 (Lesions Simulating Disease resistance1) protein, which has previously been implicated in the hypersensitive response [14]. The significance of this finding remains to be determined.

Biochemical evidence suggesting a role for cysteine proteases in plant PCD has been slowly accumulating. There have been two recent reports of a caspase-3-like activity in tobacco suspension cells and in barley embryonic suspension cells. In the first report, the levels of poly(ADP-ribose) polymerase (PARP), a substrate of caspase-3 in animal cells,

Figure 2
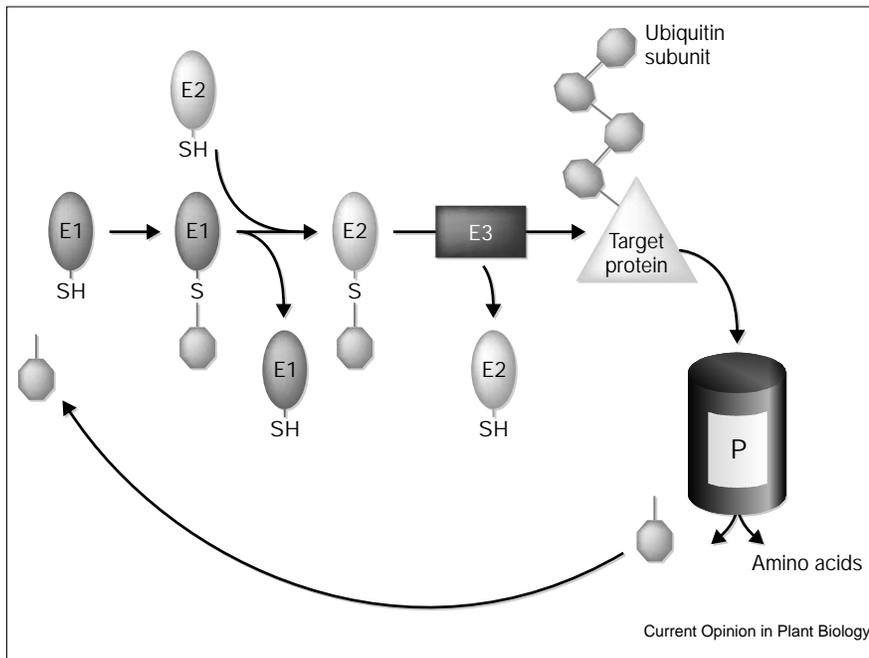
The ubiquitin protein conjugation pathway. Ubiquitin is conjugated to protein substrates via a pathway that includes a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase (E3). A protein with a chain of at least four ubiquitin subunits is recognized by the proteasome and degraded. The ubiquitin subunits are removed from the substrate by a ubiquitin-specific protease and recycled.

were determined during PCD of tobacco cells [15•]. The investigators found that PARP is cleaved before the occurrence of DNA fragmentation in the dying cells. This cleavage is reduced by an inhibitor of caspase-3, suggesting that a caspase-like enzyme performs the cleavage. In the second report, the presence of a caspase-3-like activity in barley was suggested by showing that a caspase-3-specific substrate, Ac-DEVD-AMC, was cleaved when added to extracts from embryonic suspension cells [16•]. Furthermore, this activity was suppressed by a caspase-specific inhibitor, but not by more general protease inhibitors. In the future, it should be possible to determine if these activities are associated with one or more of the metacaspases identified by Uren *et al.* [13••].

Cellular regulation by the ubiquitin-proteasome pathway

The ubiquitin-proteasome pathway (Figure 2) has been implicated in the degradation of diverse proteins in eukaryotes [17]. In many instances, the substrate protein has a role in signaling or cell cycle control. Ubiquitin–protein conjugation requires the sequential activity of three enzymes or protein complexes called the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin-protein ligase (E3). In most eukaryotes, there are a small number of similar E1 isoforms without apparent functional specificity. The E2 family is larger, with at least 36 isoforms in *Arabidopsis* [18]. Different E2 enzymes have specialized functions, presumably because they are localized to different cellular compartments and interact with varied E3s. The E3 proteins are responsible for directly interacting with substrate proteins and are very

diverse. The five main classes of E3s are the HECT-domain proteins, Ubr1-like E3s (which are responsible for amino-end-rule substrates), the anaphase-promoting complex (APC), the S-phase kinase-associated protein1 (Skp1)-cullin-F-box (SCF) type E3s, and monomeric RING-H2 E3s. Members of each class are present in plants [18].

SCF-type E3s have been implicated in a variety of signaling processes in plants (reviewed in [19]). The SCF complex was originally identified in yeast and consists of four subunits — Skp1, Cdc53 (Cell division cycle53) (or cullin in other species), Rbx1 (Ring box protein1) (also called ROC1 [Regulator of Cullins1] and Hrt1 [High-level expression reduces Ty3 transposition]), and an F-box protein. The F-box protein functions as the receptor for the complex, interacting with substrate proteins and bringing them into close proximity with the E2. The first SCF identified in plants was SCF[TIR1] (TIR1, TRANSPORT INHIBITOR RESPONSE1). This complex functions in auxin response, probably by mediating the degradation of inhibitors of the response [20]. One potential class of substrates for SCF[TIR1] are the Aux/IAA (Auxin/INDOLE ACETIC ACID) proteins. These proteins are extremely unstable and, at least under some circumstances, can function as repressors of auxin-regulated gene expression [21]. Genetic studies have shown that mutations in a short conserved sequence, called domain II, in several members of the Aux/IAA family (e.g. AXR3 [AUXIN RESISTANT3]/IAA17 and SHY2 [SHORT HYPOCOTYL2]/IAA3) result in defects in auxin response [22,23]. Most recently, the *axr2-1* mutation, which is known to confer high levels of auxin resistance, was shown to affect a residue in domain II of

the IAA7 protein [24••]. It has been proposed that these mutations may disrupt auxin response by increasing the stability, and consequently the levels, of the mutant proteins [25,26]. Support for this idea was obtained in an important study that localized the determinant(s) of Aux/IAA instability to domain II [27••]. This work also showed that the amino-acid substitutions present in the *axr3-1* and *axr3-3* alleles conferred increased stability upon a pea IAA6-luciferase protein. Thus, degradation of the Aux/IAA proteins appears to be important for auxin response. Experiments to determine if these proteins are indeed substrates of SCF^TIR1 are underway.

Studies in animal and fungal systems indicate that a single SCF is typically responsible for the ubiquitination of several unrelated substrates. Similarly, SCF^TIR1 may regulate the stability of a variety of proteins that function in auxin-regulated processes. The recent identification of proteins that are involved in auxin transport provides an opportunity to investigate the role of proteolysis in this aspect of auxin biology [28]. The PIN2 [PIN-FORMED2]/EIR1 [ETHYLENE INSENSITIVE ROOT1] protein functions as an auxin efflux carrier in *Arabidopsis* roots. In a recent study of *EIR1* expression using promoter and translational-β-glucuronidase (GUS) fusions, Sieberer *et al.* [29•] showed that the levels of EIR1 protein decreased upon auxin treatment. This change was apparently unrelated to a reduced transcription of the *EIR1* gene. Further, these investigators showed that this affect was abolished in the *axr1* mutant. Because AXR1 appears to regulate the activity of SCF^TIR1 [19,30], it is possible that EIR1 itself is a substrate for the SCF. It is also possible, however, that EIR1 instability is regulated downstream of SCF^TIR1, perhaps by another E3 or a different proteolytic system. As the *axr1* mutants are deficient in virtually all auxin responses [31], it follows that EIR1 degradation, which appears to be an auxin-dependent event, would be affected by the *axr1* mutation.

Protein degradation is an important aspect of cell cycle regulation. Recent genetic studies in plants have implicated SCF-type E3s in another cyclical process, circadian rhythm. Mutations in two related *Arabidopsis* genes, called *ZEITLUPE* (*ZTL*) and *FKF1* (*Flavin-binding, Kelch repeat, F box 1*), result in a defect in circadian rhythm [32••,33••]. ZTL and FKF1 each have an F-box, suggesting that they are subunits of an SCF and that a component(s) of the circadian machinery is a substrate for ubiquitin-mediated degradation. It will be interesting to learn how these SCFs are regulated and the identity of their substrates.

With the exception of the HECT-domain E3s, members of each class of E3s contain a subunit with a RING-finger motif. This subunit is thought to interact with the E2 enzyme to facilitate the transfer of ubiquitin from the E2 to a lysine on the target protein. In the case of the SCF and

APC, substrate recognition is accomplished by other subunits in the complex. In a growing number of cases, however, the RING-finger protein appears to act as a monomeric E3 [34]. The best known examples of this type of E3 are members of the Cbl family of proteins, which are involved in the ubiquitination of receptor tyrosine kinases in animals. Most recently, the Parkin protein, which is associated with hereditary Parkinson's disease, has been shown to have E3 activity [35]. In plants, the activity of the CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) protein plays a key role in the repression of photomorphogenesis in the dark (Figure 3). COP1 consists of a series of WD40 repeats; a coiled-coil domain, which is involved in dimerization; and a RING-finger motif. These components suggests that the protein may function as an E3. In a series of elegant studies, Deng and his colleagues [36,37••,38•] showed that showed that COP1 probably targets the bZIP (basic region and leucine zipper) protein HYPOCOTYL5 (HY5) for degradation in the dark. Earlier studies had shown that COP1 is present in the nucleus in the dark but is excluded from the nucleus in the light [36]. In contrast, HY5 accumulates in the light and stimulates the transcription of light-responsive genes. Recently, Deng and coworkers [37••] have shown that in the dark, HY5 interacts with COP1 in the nucleus and is degraded. Further, HY5 is phosphorylated within the COP1-interaction domain by a light-regulated kinase that may be a caseine kinase II [38•]. In the light, this kinase activity is inhibited. The phosphorylated form of HY5 does not interact with COP1 as well as the unphosphorylated form does, and is not as physiologically active. Thus, activity of HY5 is regulated by two linked mechanisms. In the dark, HY5 levels are reduced by COP1-mediated degradation. The remaining HY5 protein is converted to the less active phosphorylated form by the kinase. In the light, HY5 is stabilized when COP1 is translocated to the cytoplasm and accumulates in the more active unphosphorylated form. Both of these events are mediated by multiple photoreceptors [38•,39].

AvrBsT is a SUMO protease
One of the most compelling reports published in the past year involves two related bacterial proteins: YopJ (*Yersinia* outer protein J), a virulence factor from *Yersinia pestis* (which is responsible for Black Death), and AvrBsT, an avirulence protein from the plant pathogen *Xanthomonas campestris* [40••]. Both of these proteins appear to cleave the ubiquitin-related protein SUMO (Small Ubiquitin-Related Modifier) from SUMO-modified proteins. SUMO is a highly conserved protein that is conjugated to other proteins in a manner similar to ubiquitin [41]. Unlike ubiquitin, however, SUMO does not target proteins to the proteasome. Instead SUMOylation appears to have diverse functions. Both ubiquitin and SUMO can be precisely removed from target proteins by specific hydrolases, and this is where YopJ and AvrBsT come into the picture. In a beautiful study, Orth *et al.* [40••] noted that the YopJ protein has some sequence similarity with ubiquitin and SUMO hydrolases. Transfection of animal cells with *YopJ*

Figure 3
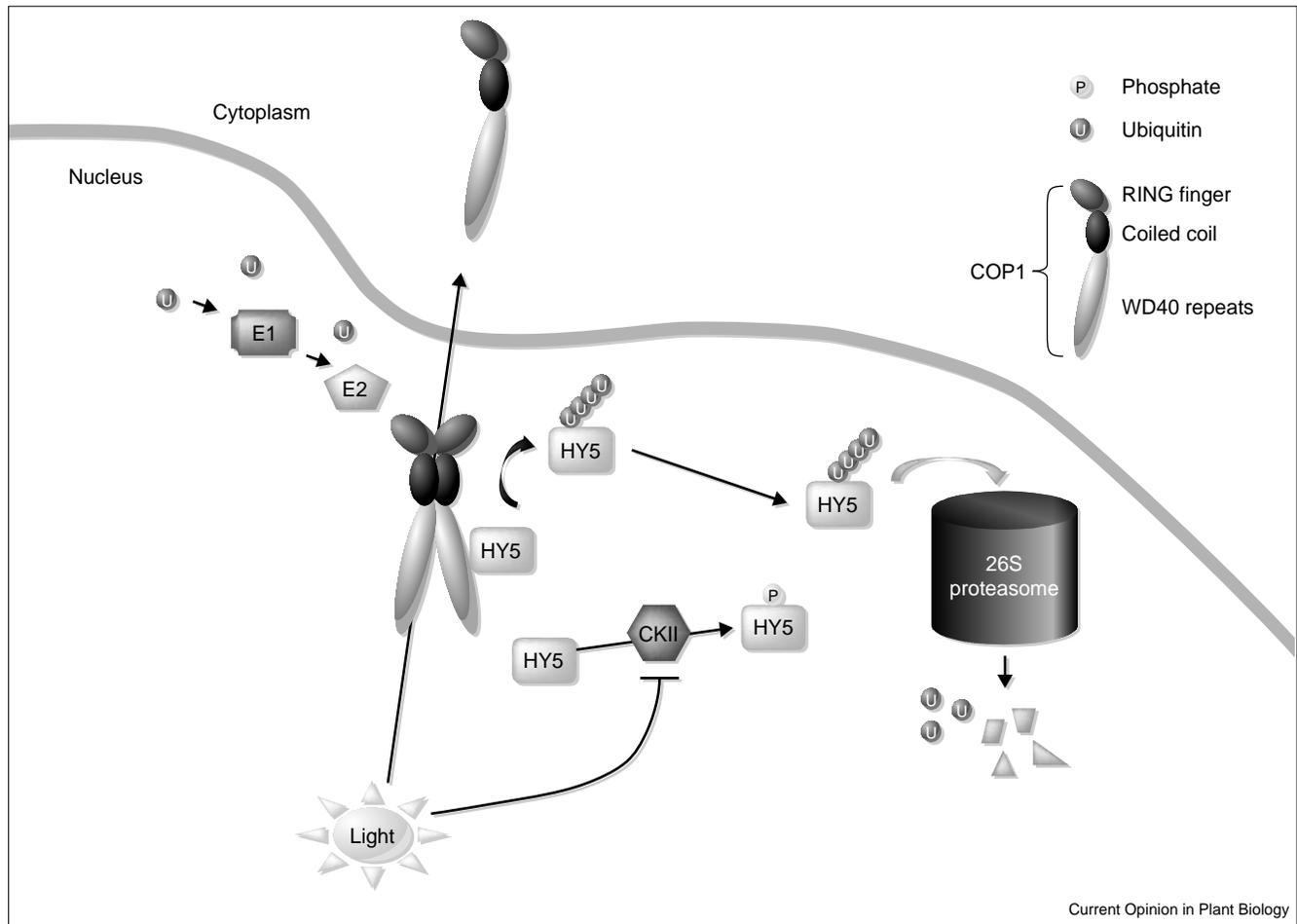
Model for COP1 regulation of HY5 activity. In the dark, COP1 interacts with unphosphorylated HY5 (the more active form), resulting in its ubiquitination and degradation. A kinase activity, possibly caseine kinase II (CKII), ensures that all residual HY5 stays in its less active phosphorylated form. Light exposure has two effects that result in increased HY5 activity. First, COP1 is translocated to the cytoplasm. Second, HY5 is preferentially accumulated in its more active unphosphorylated form due to a light-triggered reduction of kinase activity. This figure was prepared with the assistance of Xing-Wang Deng.

resulted in a decrease in the amount of SUMOylated proteins, presumably because YopJ was removing SUMO from substrates. Mutations in YopJ that eliminate the proteolytic activity also suppressed the virulence conferred by the protein. Remarkably, when the same mutation was introduced into AvrBsT, the mutant protein no longer elicited a hypersensitive response on *Nicotiana benthamiana* leaves. The authors conclude that both disease processes, infection of mammalian cells by *Y. pestis* and the hypersensitive response induced by *X. campestris*, depend on the removal of SUMO from key signaling proteins in the cell. In the case of *Y. pestis*, YopJ appears to disrupt mitogen-activated protein kinase (MAPK) and NF-κB (Nuclear Factor-kB) signaling. It will be fascinating to learn if similar signaling pathways are involved in the hypersensitive response.

Conclusions

Exciting as these recent results are, it is clear that we have just begun to appreciate the complexity of cellular regulation by proteolysis. For example, in a 1997 study, nine novel SDS-stable proteases were identified in chloroplasts [42]. The identity and biological function of these proteases is unknown at present. Similarly, an examination of the recently completed *Arabidopsis* genome sequence reveals an extremely large number of genes that are involved in proteolysis. According to the *Arabidopsis* Genome Initiative, 337 *Arabidopsis* proteins contain an F-box and 358 a RING finger [43]. If, as seems likely, most of these proteins function as E3 enzymes (or as components of E3s), it will be some time before we understand the biological functions of all of them. In addition, this review does not touch upon the huge diversity of other proteases, such as matrix metalloproteases, processing proteases, and the proteases involved in mobilization of storage-protein reserves. During the first half-century of the molecular biology era, our focus has been on the processes of protein synthesis. In the future, the complex regulation of protein degradation is likely to demand equal attention.

Update

In a recent paper, Haussuhl *et al.* [44] report the identification of DegP2 as the protease responsible for the initial cleavage of the D1 protein into 23- and 10-kDa fragments. The DegP2 protease is encoded by a single-copy nuclear gene and is a member of the prokaryotic Deg/Htr family of serine endopeptidases. As predicted by earlier studies, DegP2 cleavage of D1 protein is GTP dependent.

Acknowledgements

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