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THE UBIQUITIN 26S PROTEASOME PROTEOLYTIC PATHWAY

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■ **Abstract** Much of plant physiology, growth, and development is controlled by the selective removal of short-lived regulatory proteins. One important proteolytic pathway involves the small protein ubiquitin (Ub) and the 26S proteasome, a 2-MDa protease complex. In this pathway, Ub is attached to proteins destined for degradation; the resulting Ub-protein conjugates are then recognized and catabolized by the 26S proteasome. This review describes our current understanding of the pathway in plants at the biochemical, genomic, and genetic levels, using *Arabidopsis thaliana* as the model. Collectively, these analyses show that the Ub/26S proteasome pathway is one of the most elaborate regulatory mechanisms in plants. The genome of *Arabidopsis* encodes more than 1400 (or >5% of the proteome) pathway components that can be connected to almost all aspects of its biology. Most pathway components participate in the Ub-ligation reactions that choose with exquisite specificity which proteins should be ubiquitinated. What remains to be determined is the identity of the targets, which may number in the thousands in plants.

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INTRODUCTION

All aspects of a plant's life are controlled by the regulated synthesis of new polypeptides and the precise degradation of preexisting proteins. Via this "protein cycle," up to 50% of the total protein is replaced by plants every week (152). Although we have long recognized the intricacies surrounding the transcriptional and translational events responsible for synthesis, only recently have we begun to appreciate the catabolic half of this cycle. This breakdown plays an important housekeeping role by removing abnormal proteins and by maintaining the supply of free amino acids during growth and starvation (153). It is also essential for most, if not all, aspects of cellular regulation by removing rate-limiting enzymes and dismantling existing regulatory networks as a way to fine-tune homeostasis, adapt to new environments, and redirect growth and development (for reviews see 69, 153, 154).

Our growing appreciation of proteolysis is mainly attributed to our recent understanding of the ubiquitin (Ub)/26S proteasome pathway, arguably the dominant proteolytic system in plants. In this pathway, the 76-amino acid protein Ub serves as a reusable recognition signal for selective protein turnover. Polymers of Ub are covalently attached to protein targets using a three-step (E1 $\rightarrow$ E2 $\rightarrow$ E3) conjugation cascade that detects specific ubiquitination signals. Targets can be in the cytoplasm, in the nucleus, on membrane surfaces that face these compartments, or even from the endoplasmic reticulum (ER) following their retrograde transport back to the cytoplasm. The resulting ubiquitinated (or ubiquitylated) proteins are then degraded by the 26S proteasome with the concomitant release of the Ub moieties for reuse. Via this cycle, the Ub/26S proteasome pathway effectively removes abnormal proteins and most short-lived regulatory proteins, thereby influencing most, if not all, intracellular events (70, 110, 159). For plants in particular, the numerous ubiquitin/26S proteasome pathway components identified [>5% of the *Arabidopsis thaliana* proteome (154)] suggest that this catabolic pathway rivals transcription and protein phosphorylation as the main regulators of plant cell functions.

The purpose of this review is to update the reader on our current understanding of the Ub/26S proteasome pathway and its functions within plants, using *Arabidopsis* as the main example. For a historical perspective, see earlier reviews on plant protein degradation (e.g., 76, 97, 152). By comparison, one can see how our view of proteolysis has matured from a few proteases to the highly sophisticated recognition schemes incorporated within the Ub/26S proteasome pathway. We also encourage tapping the wealth of data from yeast (*Saccharomyces cerevisiae*) and

animals, given the strong conservation of the pathway among the three kingdoms (for reviews see 70, 110, 159).

GENERAL FEATURES OF THE UBIQUITIN/26 PROTEASOME PATHWAY

The Ubiquitin Protein

As the name implies, Ub is nearly ubiquitous, being present in all eukaryotic species examined. It is also the most structurally conserved protein yet identified; its amino acid sequence is invariant in all higher plants and differs from yeast Ub by only two residues, and from animal Ub by three residues (12). At the three-dimensional level, the bulk of the protein assumes a compact globular shape with a five-strand mixed β sheet forming a cavity into which an α helix fits diagonally (Figure 1*a*, see color insert); this structure is now referred to as the "Ub fold" (153). Numerous intramolecular hydrogen bonds provide Ub with a remarkable stability, presumably to prevent denaturation during the conjugation/target-degradation cycle, and thus encourage Ub recycling. Protruding from the Ub fold is a flexible C-terminal extension that terminates with an essential glycine. The carboxyl group of this glycine interacts covalently with E1s, E2s, and some E3s and ultimately participates in the bond that connects Ub to its targets (Figure 1*A*).

Ub is also unique among plant proteins because it is synthesized from fusion-protein precursors (12). Members of the *UBQ* family express either Ub polymers, in which multiples of the 228-bp coding region are concatenated head-to-tail, or Ub fusions, in which a single Ub-coding region is attached to the 5′ end of another coding region (12). The Ub moieties are then released from the initial translation products by deubiquitinating enzymes (DUBs), a novel protease family that cleaves precisely after the terminal glycine (161). The polyUb genes encode varying numbers of Ub repeats; in *Arabidopsis*, for example, repeat genes containing 3, 4, 5, and 6 Ub-coding units are present (12). The Ub-fusion genes encode either of two different ribosomal subunits or the Related to Ub (RUB)-1 protein fused to the C terminus of Ub (13, 113). Like Ub, these three polypeptides also become functional after DUBs release them. All the active Ub-coding genes encode the same 76-amino acid Ub sequence. This remarkable conformity, which for *Arabidopsis* involves 28 Ub-coding units, is likely maintained by continual gene conversion events (12, 136).

In a typical cell, mRNAs for at least several of the polyUb genes and all three types of Ub-fusion genes accumulate, thus providing the cell with an ample supply of Ub monomers (12). Increased expression is evident during rapid growth and stress, consistent with the role of the Ub/26S proteasome pathway in removing short-lived regulatory proteins and abnormal polypeptides. *UBQ* promoters are highly active and have been exploited to drive the transgenic expression of other proteins, especially in cereals (20). Ub is also effective for augmenting the

accumulation of recalcitrant proteins by expressing them as C-terminal fusions with Ub (74).

The Ubiquitin Conjugation Cascade

Free Ubs are attached to appropriate intracellular targets by an adenosine triphosphate (ATP)-dependent E1 → E2 → E3 conjugation cascade (Figure 1*B*). The cascade begins with an E1 (or Ub-activating enzyme) catalyzing the formation of an acyl phosphoanhydride bond between the adenosine monophosphate (AMP) moiety of ATP and the C-terminal glycine carboxyl group of Ub, and then binding the Ub directly via a thiol-ester linkage between the Ub glycine and a cysteine in the E1. This activated Ub is transferred to a cysteine in an E2 (or Ub-conjugating enzyme) by transesterification. Finally, the Ub-E2 intermediate delivers the Ub to the substrate using an E3 (or Ub-protein ligase) as the recognition element. The end product is a Ub-protein conjugate in which an isopeptide bond is formed between the C-terminal glycine of Ub and one or more lysl ε-amino groups in the target.

Depending on the substrate and/or the E2/E3 complex, several types of conjugates are possible, each of which confers a distinct fate. In some cases, only a single Ub is added; this monoubiquitination can direct proteolytic targets to the lysosome/vacuole for turnover (71) or modify transcription (5). More often a polymer of Ubs is attached, built by reiterative rounds of conjugation using a lysine within the previously bound Ub as the acceptor site (110). How these polymers are generated is not yet clear. They could either be preassembled in a free form and then attached en masse to the target and/or be processively assembled directly on the target by the E3 with or without the help of additional factors. All seven Ub lysines can be used to form polyUb chains (Figure 1*A* and 108). The most abundant in plants are Lys48-linked polyUb chains, which are preferred by the 26S proteasome (146). Other than containing an accessible lysine, the sequence surrounding the Ub attachment site is highly variable (108, 110).

E1s OR UB-ACTIVATING ENZYMES E1s initiate the conjugation cascade and have little impact on target specificity. They are single ubiquitously expressed polypeptides of ~1100 amino acids that contain a positionally conserved cysteine that binds Ub and a nucleotide-binding motif that interacts with either ATP or the AMP-Ub intermediate (66). Because of their high catalytic efficiencies, low-enzyme concentrations are sufficient to generate the pool of activated Ub needed by downstream reactions (110). *Arabidopsis* expresses only two E1 isoforms, one of which may be nuclear localized (66).

E2s OR UB-CONJUGATING ENZYMES Plants express a large family of E2 isoforms. For example, at least 37 E2 (or *UBC*) genes that cluster into 12 subfamilies are in the *Arabidopsis* genome (7, 153). E2s are easily identified by a conserved 150-amino acid catalytic core that surrounds the active-site cysteine buried within a shallow groove (61). Some E2s contain only this core whereas others have N- and C-terminal extensions that presumably assist in target recognition, association

with appropriate E3s, and/or localization. E1s and E3s dock with E2s by the same motif, indicating that E2s must shuttle between these two partners during their reaction cycle (110). Presumably, multiple E2 isoforms are needed to guarantee the equitable distribution of activated Ub to the vast array of E3s.

Individual E2 isoforms in yeast and animals have distinct functions [e.g., cell cycle regulation, DNA repair, and degradation of ER translocated proteins (70, 110)], presumably because of their interactions with specific E3s. Although numerous plant E2 subfamilies have been characterized biochemically (24, 153), little is known about their functions and specificity in vivo, primarily because plant E2 mutants have not yet been described. Some plant E2s have yeast orthologs based on protein sequence, enzymatic assays, and/or complementation (7, 24, 153). For instance, one *Arabidopsis* E2 subfamily is structurally related to yeast Ubc6 and thus may be involved in degrading ER retrotranslocated proteins (85). Members of the *Arabidopsis* UBC8 family are the most influential. They are widely expressed, responsible for much of the E2 activity in crude plant extracts, and work with most E3 types in vitro (e.g., 8, 52, 63, 125, 166).

Plants, like other eukaryotes, also express a family of Ub-E2 variants (UEVs) (7, 137). UEVs contain the conserved E2 core domain but lack the active-site cysteine and a priori cannot participate directly in Ub conjugation. The yeast UEV Mms2 works in a heterodimer combination with a bona fide E2 Ubc13, and a subset of E3s to assemble polyUb chains linked via Lys63 (145). Attachment of these chains confers nonproteolytic function(s), which in one case promotes DNA repair.

E3s OR UB-PROTEIN LIGASES As the last components in the Ub-conjugation cascade, E3s are responsible for identifying the many proteins that should be ubiquitinated. Consequently, they are the most numerous and diverse factors of the ubiquitination cascade. For example, the *Arabidopsis* genome contains more than 1300 genes that encode putative E3 subunits, with one family containing almost 700 members (50, 154).

Four E3 types have been described thus far in plants, based on subunit composition and mechanism of action [Homology to E6AP C Terminus (HECT), Real Interesting New Gene (RING)/U-Box, a complex of Skp1, CDC53, and F-box protein (SCF), and anaphase-promoting complex (APC)], and it is likely that more exist (4, 14, 39, 50, 84, 171). In several cases, these E3s have been confirmed in vitro to possess ligase activity by their ability to stimulate ATP-dependent ubiquitination in the presence of E1 and an appropriate E2 (8, 63, 125, 135, 166, 171). The X-ray crystallographic structures of several representatives from animals have been determined. These structures identified the E2-binding site and suggested a mechanism for Ub transfer for three (HECT, RING, and SCF) (104, 105, 148, 165, 181).

HECT E3s are single polypeptides easily recognized by the presence of a conserved 350-amino acid C-terminal region called the HECT domain first detected in the founding member, human E6AP (39). They are typically large proteins (>100 kDa): the seven HECT E3s in *Arabidopsis* range in size from 96–405 kDa

(8, 39). Unlike other E3s, they form a Ub-E3 thiol-ester intermediate with a unique cysteine in the HECT domain, which then serves as the proximal Ub donor during the ligation reaction. The region upstream of the HECT domain contains additional motifs that participate in target recognition, Ub binding and/or localization, including Armadillo, IQ calmodulin-binding, C-type lectin-binding, transmembrane, Ub-interacting motif (UIM), Ub-associated (UBA), and Ub-like (UBL) domains (39). The UIM, UBA, and UBL sequences, in particular, appear in several other contexts related to Ub metabolism and work by either helping recognize Ub [UIM and UBA (39, 72)] or by mimicking its structure [UBL (41)]. Little is known about the functions of plant HECT E3s; one *Arabidopsis* isoform is necessary for trichome development (39).

The RING/U-Box E3s are a loosely defined collection of polypeptides bearing either a signature RING-finger motif or a structurally related derivative called the U-Box. Sequence analyses in plants have identified large families of each type. The *Arabidopsis* genome encodes approximately 480 RING finger–containing proteins and 64 proteins with a U-Box motif (4, 84, 171; H. Hauksdottir & J. Callis, unpublished work). For the RING E3s, the 70—amino acid finger is a cross brace formed by an octet of cysteines and histidines that bind zinc in either a $C_3H_2C_3$ (RING-H2) or a $C_3H_1C_4$ (RING-HC) configuration (182). The U-Box exploits electrostatic interactions instead of metal ion chelation to stabilize a RING finger–like structure (104). The RING/U-Box serves as a Ub-E2 docking site that allosterically activates transfer of the Ub to substrate lysine(s). Numerous other motifs (e.g., WW, WD-40, and ankryin) are present, which presumably endow target specificity (84, 171). Genetic analyses of several indicate that they play diverse roles in plant physiology, including photomorphogenesis (73, 106, 125), auxin signaling (166), cold sensing (91), self incompatibility (135), wax biosynthesis (62), and removal of misfolded polypeptides (171).

SCF E3s consist of a complex of at least four polypeptides. The founding member was named SCF based on three of its core subunits: SKP1, CDC53 (or Cullin), and an F-Box protein (29). Subsquently, the fourth subunit RBX (or ROC1 and HRT1) was discovered and found to contain a RING H2–type domain. Like RING/U-Box E3s, SCF E3s function as scaffolds that bring together the activated Ub-E2 complex and the target to promote conjugation without forming an E3-Ub intermediate. The Cullin-RBX-SKP1 subcomplex provides the Ub-transferase activity and a multitude of F-Box proteins confer target specificity (29). F-Box proteins contain a signature F-Box motif near their N-termini, which anchors the subunit to the rest of the SCF complex by interacting with SKP1. Located at their C-termini is one of several protein-protein interaction motifs (e.g., leucine rich repeats (LRRs), WW, tetratricopeptide repeats (TPRs), Kelch, or Armadillo) that presumably identifies appropriate targets (50). In many cases, substrate phosphorylation is an important prerequisite (29) intimately connecting these two protein modification events.

Several accessory factors, including SGT1 and CAND1, also associate loosely with SCF E3s (82, 94). Genetic analyses in yeast suggest that they control SCF

activity, possibly by promoting complex assembly with appropriate targets (SGT1), or stimulating disassembly following target ubiquitination (CAND1). The Cullin subunit is also activated by the reversible attachment of RUB1 a protein structurally related to Ub (see below). Some F-Box proteins can also direct auto-ubiquitination, possibly as a way to negatively regulate SCF E3 levels in the absence of substrate (110).

Unlike other eukaroytes, plants can synthesize an amazing number of SCF complexes. For example, whereas yeast and human genomes contain 14 and 74 F-Box protein genes, respectively (124), the *Arabidopsis* genome contains almost 700 (50). This diversity coupled with the presence of two RBX1 subunits (55, 90), at least five Cullins [called CUL1, 2, 3a, 3b, and 4 (128)], and 21 possible SKPs [called ASKs in *Arabidopsis* (42)] could generate an infinite array of distinct SCF ligases. However, directed yeast two-hybrid analyses of ASKs with representative F-Box proteins imply that a more limited number of combinations are actually assembled, which suggests that SCF E3s have a hierarchical organization defined by specific protein pairings (50, 115). It is also possible that members of the Cullin and SKP families interact with entirely new sets of substrate recognition factors to further expand specificity. For example, in animals, CUL2 forms part of the VBL E3 complex (101), CUL3 interacts with proteins that contain a Broad-Complex, Tramtrack, and Bric-a-brac (BTB) motif (170), and CUL4A recruits the substrate recognition factors DDB2 and CSA1 (58) to generate alternative SCF-like complexes. Preliminary data indicate that plants also assemble an array of CUL3/BTB complexes (D. Gingerich & R.D. Vierstra, unpublished data). As expected from the number of F-Box subunits, SCF E3s participate in a broad range of cellular events in plants (see below and 69, 154).

The APC is the most elaborate E3 type, with the core particle containing at least 11 subunits. It was first identified as essential for degrading mitotic cyclins and subsequently demonstrated to control the half-life of other factors crucial for mitotic progression and exit (64). *Arabidopsis* orthologs for almost all APC subunits have been detected, indicating that a similar complex exists in plants (14, 15). Most of these subunits are encoded by single genes, implying that, unlike the RING/U-Box and SCF E3s, a small set of APC isoforms is assembled. Two of the APC subunits, APC2 and APC11, are related to CUL1 and RBX1 of the SCF E3 complex, respectively. Presumably, they act similarly in helping to scaffold the remaining subunits (APC2) and by binding the Ub-E2 intermediate (APC11). In this case, the *Arabidopsis* E2 is the UBC19-20 subfamily (24). Consistent with the crucial role of the APC in the cell cycle, mutations affecting several *Arabidopsis APC* genes block cell division (10, 15).

Two target recognition subunits for APC have been identified, CDC20/Fizzy and CDH1/Fizzy-related, that are presumably interchangeable in the complex (64). In animals and yeast, these factors use a WD40 domain to bind substrates bearing either D-Box (CDC20) or KEN Box (CDH1) degradation signals. Whereas yeast encodes single Cdc20 and Cdh1 subunits, small families of each can be found in *Arabidopsis* [six and three, respectively (14)] and *Medicago truncatula* (17),

suggesting that the plant APC recognizes a greater repertoire of targets. In yeast and metazoans, APC action is controlled by phosphorylation/dephosphorylation of both the complex and targets, often in a cell cycle–dependent manner (64).

The 26S Proteasome

The 26S proteasome is a 2-MDa ATP-dependent proteolytic complex that degrades Ub conjugates (for reviews see 65, 157). Although most of our current understanding is derived from the yeast and mammalian particles, work on the plant complex, particularly from *Arabidopsis* and rice, indicates a similar design (44, 45, 129, 176). However, genomic and genetic analyses revealed that multiple isoforms of the 26S proteasome are assembled, which suggests that plants have expanded its capabilities (129, 176).

The 26S proteasome contains 31 principal subunits arranged into two subcomplexes, the 20S core protease (CP) and the 19S regulatory particle (RP) (Figure 2, see color insert). The CP is a broad spectrum ATP- and Ub-independent protease. It is a cylindrical stack created by the assembly of four heptameric rings. The two peripheral rings are composed of seven related α subunits and the two central rings are composed of seven related β subunits in a $\alpha_{1-7}/\beta_{1-7}/\beta_{1-7}/\alpha_{1-7}$ configuration. X-ray crystallographic analyses of the yeast and mammalian CPs revealed a large central chamber that houses the protease active sites provided by the β1, β2, and β5 subunits (60, 142). They belong to the NTn hydrolase family that uses an N-terminal threonine as the active-site nucleophile; this residue is exposed following cleavage of a propeptide. The β1, β2, and β5 subunits generate peptidylglutamyl peptide—hydrolyzing, trypsin-like, and chymotrypsin-like activities, respectively, thus imbuing the CP with the capacity to cleave most, if not all, peptide bonds (157). Like their yeast and animal counterparts, the active sites of the plant CP are very sensitive to the 26S proteasome inhibitors, MG115, MG132, lactacystin, and epoxomycin (176). A small channel formed by each α-subunit ring restricts access to this chamber such that only unfolded proteins may enter (Figure 2). Flexible N-terminal extensions of the α subunits gate this channel to control substrate entry and possibly product exit (59, 65). In this way, the CP spatially separates proteolysis from the cellular milieu and restricts degradation to only those polypeptides that are deliberately unfolded and imported.

The RP associates with one or both ends of the CP and confers both ATP dependence and a specificity for Lys48-linked polyUb chains to the particle (65, 157). Although little is known about its three-dimensional structure, a protein interaction map was recently developed (46). The RP is composed of 17 core subunits that can be further divided into the Lid and Base subcomplexes (Figure 2) (46, 54). The Base sits directly over the α-ring channel; it contains a ring of six related AAA-ATPases (RPT1-6) and three non-ATPase subunits (RPN1, 2, and 10). The Lid contains the remaining non-ATPase subunits (RPN3, 5–9, and 11–12).

Collectively, the RP assists in recognizing and unfolding appropriate substrates, removing the covalently bound Ubs, opening the α-ring gate, and then directing

the unfolded polypeptides into the lumen of the CP for breakdown (Figure 2). Currently, the functions of only a portion of the RP subunits are known. The RPT ring contacts the α-ring of the CP and presumably uses ATP hydrolysis to facilitate channel opening and protein unfolding (65). Additional functions are likely considering that specific RPTs interact with polyUb chains [RPT5 (88)] and a variety of other non-RP proteins, including E3s (e.g., 167). RPN11 is a Jab1/MPN domain-associated metalloprotease (JAMM) with DUB activity; it helps disassemble polyUb chains during target degradation (149). RPN1 can bind UBL domains that might help shuttle substrates to the 26S proteasome (41). RPN10 helps tether the Lid to the Base using an N-terminal von Willebrand Factor (vWF) A-like domain as the interaction site (46). It also contains a C-terminal UIM that binds Lys48-linked polyUb chains, and consequently might function as a receptor for ubiquitinated proteins (46, 47). However, polyUb binding by RPN10 is not essential in yeast, *Physcomitrella patens*, and *Arabidopsis* (46, 53, 132), implying that RPN10 is not the main Ub-recognition element. Some remaining RP subunits may have target-specific functions that possibly include recruiting specific E3s or carrier proteins that deliver ubiquitinated cargo to the 26S proteasome. For example, *Arabidopsis* RPN12a and RPN5a/b participate in cytokinin responses and cell proliferation, respectively, suggesting that they help identify a subset of 26S proteasome targets controlling these processes (131; J. Smalle & R.D. Vierstra, unpublished data).

A host of other proteins more loosely bind to the 26S proteasome, possibly at substoichiometric levels, suggesting that the 26S proteasome is actually the stable core of an even larger particle (93, 150). In yeast, extra proteins include the HECT-E3 Hul5, the DUB Ubp6, and Ecm29 that helps tether the CP to the RP. Orthologs to each of these are encoded in the *Arabidopsis* genome. In plants, two proteins kinases (one related to SNF-1 and the other a member of the calcium-dependent kinase family) interact with the particle possibly as a way to modify its activity (42, 92). The animal (and likely plant) RP also contains an additional DUB, whose activity may be important for trimming polyUb chains during substrate degradation (89; P. Yang & R.D. Vierstra, unpublished work).

Substitutions and modifications of the core 26S proteasome may also affect activity/specificity. The most dramatic example is the mammalian "immunoproteasome," which is created by substituting the three active-site β1, β2, and β5 subunits of the CP with homologs and by replacing the RP with an unrelated PA28 complex (or 11S regulator) (28). Integrating these alternative subunits changes the catalytic specificity of the CP and makes the holoenzyme work independently of the Ub tag, thus creating a protease efficient in antigen presentation. Although plants do not appear to contain PA28, they do express another CP activator called PA200 that assists in DNA repair (144). Similar to immunoproteasomes, plants may assemble alternative 26S proteasomes by incorporating nonredundant subunit isoforms (131; J Smalle & R.D. Vierstra, unpublished data). For example, in *Arabidopsis* most of the 26S proteasome subunits are encoded by two genes; both

protein products for many of these pairs can be detected in purified 26S proteasome preparations (44, 45, 176). An intriguing possibility is that plants synthesize an array of 26S proteasomes that are deliberately engineered to handle specific substrates. Additionally, various subunits may be modified by phosphorylation, which could in turn affect assembly and/or activity of the 26S particle (116).

Although most substrates of the 26S proteasome likely require prior ubiquitination, Ub-independent routes are possible. For instance, several mammalian proteins are substrates in the absence of a polyUb signal (151). In some cases, target delivery could involve a collection of proteins bearing a UBL motif, including RAD23, DSK1 and 2, and BAG1 (41), some of which have orthologs in plants (155). These carrier proteins could bind and deliver substrates to the 26S proteasome by interaction of their UBL sequence with one or more Ub receptors, such as RPN1 and RPN10.

The Lid of the 26S proteasome appears to be evolutionarily related to the eIF3 and COP9/signalosome (CSN) complexes (126, 158). Like the Lid, they both contain eight synonymous subunits that scaffold together using a similar set of PCI (Proteasome, COP9, eIF3) and MPN (MPR1, PAD1, N-terminal) protein-interaction motifs (46, 54). The eIF3 complex is involved in translational control, whereas the CSN helps regulate a number of signaling pathways in both plants and animals. Even though the CSN is typically purified in a free form, preliminary data suggest that the entire CSN associates with at least part of the 26S proteasome and one or more SCF E3s to create a larger proteolytic complex (109). The *Schizosaccharomyces pombe* eIF3 subunit SUM1 can also associate with the 26S proteasome through RPN5, suggesting that these two complexes interact as well (40). An interesting possibility is that the CSN, and maybe the eIF3 complex, replace the Lid to create alternative 26S proteasomes.

The 26S proteasome is present in both the cytoplasm and nucleus of plant cells, with the highest amounts found in rapidly dividing tissues (42, 87, 92). During stress, the level of the complex increases. In yeast, this upregulation is directed by Rpn4, a transcription factor that activates the expression of most, if not all, 26S proteasome subunit genes through negative feedback control of its half-life (168). Under normal conditions, Rpn4 is rapidly degraded by the 26S proteasome thus maintaining a low rate of 26S proteasome synthesis. But in situations that overwhelm the protease with substrates (e.g., rapid growth, stress, and impaired 26S proteasome activity), Rpn4 is stabilized, thus allowing subunit synthesis to rise. A similar, coordinated transcriptional upregulation of 26S proteasome genes is evident in *Arabidopsis* when 26S proteasome activity is diminished by mutation, suggesting that a similar negative feedback regulatory system exists (176).

Deubiquitinating Enzymes (DUBs)

Like animals and yeast, plants contain a family of DUBs (159, 161). They generate free Ub moieties from their initial translation products, recycle Ubs during breakdown of the polyUb-protein conjugates, and/or reverse the effects of ubiquitination (Figure 1*B*). For example, in *Arabidopsis*, at least 30 genes can be detected that

encode potential DUBs. This collection includes two ubiquitin carboxy terminal hydrolases (UCHs), which use a catalytic triad of cysteine/histidine/aspartic acid residues; 27 Ub-specific proteases (UBPs), which are thiol proteases employing a characteristic pair of cysteine and histidine boxes; and one RPN11, which is a DUB in the 26S proteasome Lid (172; P. Yang & R.D. Vierstra, unpublished data). All DUBs tested have remarkable specificity for Ub. They recognize the proximal Ub moiety and remove almost any amino acid or peptide attached to the C-terminal glycine (e.g., 38, 172).

DUBs have been implicated in a variety of processes in animals and yeast, suggesting that individual DUBs are target specific (161). An intriguing possibility is that some DUBs can also regulate a protein's half-life by reversing the ubiquitination reaction. Two *Arabidopsis* UBP subfamilies have roles in removing damaged proteins (*At*UBP1 and 2) (172) and in recycling free Ub chains (*At*UBP14) (38). Recent studies with *Arabidopsis* UCH1 and 2 suggest that this pair participates in the auxin response (P. Yang & R.D. Vierstra, unpublished work).

Genomic Analysis of the Ub/26S Proteasome System

With the release of near-complete genomic sequences for *Arabidopsis* and rice, it is now possible to define the size and complexity of the Ub/26S proteasome pathway in plants via bioinformatic methods. For example, applying reiterative BLAST searches using consensus motifs for the various pathway components as queries, scientists have identified more than 1400 *Arabidopsis* genes that encode Ub/26S proteasome-related factors (154; R.D. Vierstra, unpublished data). This number alone argues that the pathway plays a prominent role in plant biology. When expressed as a fractional percent of the genome, these components represent >5% of the total proteome, which is more than twice that of yeast, *Drosophila*, mice, and human (124). Why have plants placed a particular emphasis on this proteolytic system? One possibility is that their sessile habit and long life spans have forced plants to adopt an additional layer of proteolytic control to more effectively regulate metabolism and development and to better survive pathogen attack and environmental stress.

When adding other factors/events that contribute to Ub/26S proteasome-mediated degradation (RUB1 conjugation, CSN, phosphorylation, etc.), the breadth of the system expands further. Consequently, it is likely that protein degradation by the Ub/26S proteasome pathway rivals transcription and protein phosphorylation in both protein complexity and physiological influence in plants. Because phosphorylation is often important for substrate recognition by E3s and for regulating the activity of various pathway components, one of its main purposes might be to control the Ub/26S proteasome pathway.

As expected, the organization of the Ub/26S proteasome pathway is hierarchical with most of the complexity residing in the E3 families that decide which proteins should be ubiquitinated. For example, whereas *Arabidopsis* contains 2 E1 genes and 37 E2 genes, it contains ~1300 genes encoding E3 components (154). In fact, the F-Box family represents the largest protein class identified thus far in plants,

with the family of predicted RING E3s close behind (50, 84; H. Hauksdottir & J. Callis, unpublished work). Like other *Arabidopsis* gene families, expansion and evolution of these E3 gene families proceeded by local and large-scale duplications followed by sequence divergence (50). For some families, related genes are often clustered, indicating that recent tandem chromosomal duplications play an important role.

Because sequence comparisons and limited genetic information suggest that many *Arabidopsis* E3s are not functionally redundant (50, 84; Table 1), it is possible that plants have an equally large number of targets. Based on estimates that 10% of total eukaryotic proteins are regulated by the Ub/26S proteasome system (70), as many as 2600 substrates might exist in *Arabidopsis*. Extrapolated further, with ~1300 different E3 components recognizing ~2600 targets, most *Arabidopsis* targets may have their own ubiquitination cascades. These devoted cascades could in turn recognize unique degradation signals as a way to confer precise specificity. The end result is that hundreds of different degradation signals may exist that have coevolved with their cognate E3s.

UBIQUITIN-RELATED PROTEINS

Since the discovery of Ub, a number of Ub-related modifiers have been identified (77, 99, 155). In plants, the current list includes RUB1 (or NEDD8), SUMO (Small Ubiquitin-related Modifier), APG8 (Autophagy-defective 8), APG12 (Autophagy-defective 12), URM (Ubiquitin-related Modifier), and HUB (Homologous to Ubiquitin), and more are possible. Although most bear little sequence identity to Ub, they all contain the Ub fold with a similar flexible C-terminal extension. These tags become attached to various targets, using mechanistically analogous ATP-dependent ligation cascades involving an E1, an E2, and sometimes an E3, and can be released by DUB-like activities in some cases. With the exception of SUMO, which can at times be assembled into polymers (37, 86), a single tag is attached. However, the numbers of targets for these tags are much smaller than those for Ub; in one case (APG8), the target is the lipid phosphatidylethanolamine, not a polypeptide (37). Like Ub, several tags have been linked to protein turnover. For example, conjugation of APG8 and 12 is important for bulk protein degradation by autophagic delivery to vacuoles (37). In contrast, RUB1 and SUMO directly impact the Ub-conjugation system (69, 86).

RUB1 shares a 75% sequence identity to Ub and assumes a near identical shape (113). Its role in the Ub/26S proteasome pathway is to reversibly modify the activity of SCF-type E3s through its covalent attachment to the Cullin subunit. It is attached to a specific lysine by a devoted conjugation pathway consisting of an E1, assembled as a heterodimer of AXR1 and ECR1 polypeptides, and an E2, RCE1 (27). During the final step, the RUB1-RCE1 intermediate associates with RBX1 possibly at the same site as Ub-E2 (55). RUB1 attachment may promote assembly of active SCF E3 complexes by forcing the dissociation of CAND1 from the Cullin (94). The CSN uses the JAMM metalloprotease activity of the CSN5/JAB1

TABLE 1 E3s and targets of the Ub/26S proteasome pathway involved in plant growth and development

	E3 (Type)[a]	Target protein(s)	References
Cell cycle			
G1/S (Rb pathway)	SKP2 (F-Box)	E2Fc	(26)
Mitosis	APC	CYCB1, CYCA3, CDC6	(16, 51)
Hormone regulation			
Auxin	TIR1 (F-Box)	AUX/IAA family	(56, 178)
Auxin	SINAT5 (Ring HC)	NAC1	(166)
Auxin	?	EIR1	(130)
Abscisic acid	?	ABI5	(95, 132)
Brassinosteroids	?	BZR1 and BZR2	(68)
Ethylene	EBF1 and 2 (F-Box)	EIN3	(50a, 60a, 110a)
Gibberellins	SLY (F-Box)	RGA	(98)
Gibberellins	GID2 (F-Box)	SLR1	(118)
Jasmonic acid	COI1 (F-Box)	RPD3b	(30)
Responses to the abiotic environment			
Light	COP1 (Ring HC)	HY5, HYH, LAF1	(73, 106, 125)
Light	CIP8 (Ring HC)	HY5, HYH	(63)
Red/far red light	?	PhyA	(21, 22)
Red/far red light	EID1 (F-Box)	?	(35)
Red/far red light	AFR (F-box)	?	(63a)
Blue light (circadian rhythm)	FKF1, LKP2 (F-Box)	?	(102, 120a)
Blue light (circadian rhythm)	ZTL (F-box)	TOC1	(96a, 133)
Circadian rhythm	?	ZTL	(81)
Heat and cold shock	AtCHIP (U-Box)	Denatured proteins	(171)
Cold signaling	HOS1 (Ring HC)	?	(91)
Responses to the biotic environment			
NIM1 pathway	SON1 (F-Box)	?	(79)
Virus spread	?	MP	(114)
Self-incompatibility	SFB (F-Box)	?	(143)
Self-incompatibility	ARC1 (U-Box)	?	(135)
Development			
Flower development	UFO/FIM/PFO/ STP (F-Box)	?	(117, 179)

(*Continued*)

TABLE 1 (*Continued*)

	E3 (Type)[a]	Target protein(s)	References
Senescence/shoot branching	ORE9/MAX2 (F-Box)	?	(134, 163)
Trichome development	UPL3 (HECT)	?	(39)
Wax biosynthesis	CER3 (RING HC)	?	(62)
Metabolic pathways			
Glycolysis	?	$PyrKin_c$	(139)
Alkaloid biosynthesis	?	TDC	(1)
N-end rule pathway	PRT1 (Ring HC)	N-end rule substrates	(111)

[a]For SCF E3s, only the F-Box mutants are included.

? Unknown.

subunit to remove RUB1 (23). Genetically dissecting the RUB1 modification cycle in *Arabidopsis* showed that it is crucial for many processes (33, 69, 126), which is consistent with the general role of RUB1 in SCF E3 activation (see below).

The consequences of SUMO modification are target dependent and include affecting protein activity and localization, and in the context of this review, protecting proteins from ubiquitination (99). SUMO is attached to an array of targets via its own conjugation system, using a heterodimeric E1 consisting of SAE1 and 2 subunits, the E2 SCE1 and one of several E3s (86). One class of SUMO ligases uses a RING finger–like motif similar to those found in Ub RING E3s. A consensus sumolyation site ΨKXE has been identified for numerous targets, where Ψ is a hydrophobic residue and K is the lysine to which SUMO is attached. In at least two mammalian examples, the same lysine can be either sumoylated or ubiquitinated, with SUMO attachment serving to block degradation by the Ub/26S proteasome pathway (100). It is possible that many Ub targets are protected similarly in plants, especially following stress (86).

FUNCTIONS OF THE UBIQUITIN/26S PROTEASOME PATHWAY

Consistent with the importance of protein turnover for cellular control, the Ub/26S proteasome pathway substantially influences much of plant biology. For cellular housekeeping, the pathway helps remove proteins that arise from synthetic errors, spontaneous denaturation, free-radical-induced damage, improper processing, and disease (70). Surprisingly, as much as 30% of initial translation products are nonfunctional and rapidly removed by the Ub/26S proteasome pathway (120). In fact, a common feature of various pathway mutants in both plants and animals is a

hypersensitivity to environmental stresses that accelerate protein denaturation, and to amino acid analogs, whose translational incorporation produces dysfunctional polypeptides (53, 70, 171, 172). For proteins that enter the secretory system, a quality control mechanism exists in the ER lumen that rejects misfolded or improperly assembled polypeptides and transports them retrograde back to the cytoplasm. These targets are then ubiquitinated by the ER-associated degradation (ERAD) pathway, consisting of a subset of E2s, the HRD1/DER3 RING E3, and the CUE1 adaptor protein in yeast (85). Some abnormal cytoplasmic proteins are first sequestered into protein aggregates called aggresomes before breakdown, presumably to store these toxic polypeptides away from the rest of the cytoplasmic milieu (83). In fact, the inability to clear these aggregates is a hallmark of several human neurological diseases, including Alzheimer's (83). It is not yet clear how these "protein graveyards" are formed but it appears to be an active process involving the microtubule network.

With respect to cellular regulation, the Ub/26S proteasome pathway is responsible for removing most short-lived regulatory proteins either constitutively or in response to internal or external changes (69, 70, 153, 154). The list includes key enzymes that direct rate-limiting steps of metabolic pathways. By conferring a short half-life to these proteins, the Ub/26S proteasome pathway can easily fine-tune metabolic flux and attenuate metabolism when the substrate is limited or the product is in excess or no longer needed. An excellent example in plants is soybean pyruvate kinase, which is removed by the pathway presumably to help control carbon partitioning (139). A number of signaling receptors are also targets upon ligand engagement, thus providing a rapid mechanism to deactivate signal transmission. For instance, phyA, a member of the phytochrome (phy) family of photoreceptors, is rapidly ubiquitinated and degraded following photoconversion to the active Pfr form (21). Most Ub/26S proteasome pathway targets activate and repress gene expression. For some of these factors, the transcriptional activation domain also serves as a signal for ubiquitination, thus directing their prompt removal after upregulation (5). Accordingly, the phenotype of many *Arabidopsis* pathway mutants can be explained by selective stabilization of key transcription factors (69, 154).

Pleiotropic Control of Homeostasis and Development

Many of our functional insights of the Ub/26S proteasome pathway are based on the characterization of an exponentially increasing collection of pathway mutants, mostly in *Arabidopsis*. Not surprisingly, mutations affecting central players often generate pleiotropic defects, or in the most severe cases, embryo lethality. Global changes in the pool of Ub conjugates, either by attenuated Ub attachment or impaired breakdown/disassembly, can also be seen (38, 53, 132). Although these extreme phenotypes confirm the importance of the pathway, they should be interpreted with caution because they may not define the full repertoire of functions for the affected protein but just those that are most crucial to the plant at its particular

developmental stage. For example, mutations affecting either the E1 (AXR1) involved in RUB1 conjugation or the CSN complex that has derubinating activity were first discovered by their auxin response and photomorphogenic defects, respectively, even though these mutants impact many Ub/26S proteasome–regulated processes (69, 126).

In addition to mutants in the RUB1 cycle, pleiotropic defects are readily apparent for mutants affecting core factors of E3 complexes, the 26S proteasome, and DUBs. For example, losing the CUL1 and RBX1 subunits of the SCF complex induces severe growth defects in *Arabidopsis* (33, 90), with a less drastic phenotype conferred by losing ASK1, the most abundant member of the *Arabidopsis* SKP family (175). Similar disruption of SGT1b, which would affect many SCF E3s, attenuates various hormone responses and pathogen defenses (2, 3, 57). A mutation affecting the *PBF1* gene encoding the β6-subunit of the *Arabidopsis* 26S proteasome CP appears to be lethal; whereas the heterozygous plants are healthy, seeds homozygous for the *pbf1* mutation cannot be recovered (J. Smalle & R.D. Vierstra, unpublished work). Loss of the *Arabidopsis* DUB that likely helps disassemble free polyUb chains (*At*UBP14) induces early embryo arrest (38, 141). The mutant embryos [*Erratum] accumulate abnormally high levels of free polyUb chains, presumably inhibiting competitively substrate competitvely degradation by the 26S proteasome (38).

Via the genetic analysis of components that act closer to the substrate, processes specifically under the control of the Ub/26S proteasome have been elucidated. Here, mutations in individual E3s have been particularly informative by generating conditional and/or less pleiotropic phenotypes. These studies have linked the pathway to most hormone responses, specific events in development, and responses to the abiotic and biotic environments (Table 1). In some cases, the phenotypes have suggested relevant targets, which have been confirmed by subsequent experiments with 26S proteasome inhibitors, in vivo tests of target half-life, and/or in vitro ubiquitination assays (56, 125, 132, 166, 178).

Cell Division

Similar to yeasts and metazoans, the plant cell cycle is driven by changes in the substrate specificity and subcellular localization of cyclin-dependent kinases (CDKs), which in turn are modulated by a collection of cyclins, CDK-activating and -inhibiting kinases, and by several CDK inhibitors (31). The half-life of many of these modulators is affected by the 26S proteasome and several distinct sets of E3s, especially the APC. Targets include the tobacco mitotic cyclins CycB1;1 and CycA3;1, and *Arabidopsis* CDC6, which is required to initiate DNA replication (15, 16, 51). Like their metazoan counterparts, tobacco mitotic cyclins have a D-Box motif commonly recognized by the APC (51). APC mutants directly connect this E3 to the plant cell cycle. For example, in *Arabidopsis*, loss of *APC2* blocks female gametogenesis, whereas a defect in an *APC3*/*CDC27* gene caused by the *hobbit* mutation impairs cell division rates and meristem differentiation (10, 15). CCS52A, the *Medicago truncatula* ortholog of the yeast APC subunit

*Erratum (14 May 2004): See online log at http://arjournals.annualreviews.org/errata/arplant

CDH1 that recognizes mitotic cyclins, has been implicated in the control of DNA endoreplication. Altering CCS52A levels profoundly affects ploidy, cell size, and ultimately root nodule organogenesis (156).

The plant cell cycle is also controlled by other E3 types, including one or more SCF E3s that act during the G1/S transition (31). CDKs regulate this transition in part through phosphorylation of the retinoblastoma protein, which then derepresses E2F transcription factors. The abundance of E2Fc, a member of the *Arabidopsis* E2F family, is regulated by an SCF E3 containing an ortholog of the human F-Box protein SKP2 (SCF^{SKP2}) (26). E2Fc is also stabilized in the *axr1-12* mutant affecting the RUB1 E1, indicating that SCF^{SKP2} activity is controlled by the RUB1 cycle (26). Supressing the SCF subunit RBX1 increases the abundance of the cyclin CYCD3, suggesting that an SCF E3 is involved in its degradation as well (90). The 26S proteasome may also contribute to plant cell cycle progression by actively associating with intracellular structures including mitotic spindles, the preprophase band, and the phragmoplast (42, 173).

Hormone Responses

The Ub/26S proteasome pathway is directly or indirectly implicated in the action of all major plant hormones (69, 154). Collectively, the data indicate that hormone signaling often leads to a secondary modification of targets that enhances either their degradation or stability. Because most known target proteins are transcriptional activators or repressors, affecting their half-lives may constitute a universal control point in hormone signaling.

AUXINS The auxin response pathway was the first to reveal a key role for the Ub/26S proteasome pathway in hormone signaling and will likely serve as a paradigm for others. In the scheme shown in Figure 3 (see color insert), auxin responses are primarily controlled by a family of short-lived nuclear-localized AUX/IAA (AUXIN/INDOLE-3-ACETIC ACID) repressor proteins that block the auxin-response transcription factors (ARFs) by heterodimerization (78). Auxin promotes AUX/IAA breakdown using a set of SCF E3s containing members of the TIR1 F-Box protein family (56) (S. Dharmasiri & M. Estelle, unpublished data). The LRR of SCF^{TIR1} directly recognizes the AUX/IAA proteins by the proline-rich Domain II, which is conserved within the AUX/IAA family (56, 112, 178). Auxin responses are profoundly altered in *axr1* mutants and mutations affecting the CSN, indicating that SCF^{TIR1} is regulated significantly by the RUB1 cycle (27, 122). SGT1 is also required because *tir1* auxin resistance is enhanced by a mutation in *SGT1b* (57). The 26S proteasome then degrades the ubiquitinated AUX/IAA proteins (112). Not surprisingly, mutants that compromise 26S proteasome activity also attenuate auxin sensitivity (131, 132).

How does auxin promote the recognition of AUX/IAA proteins by SCF^{TIR1}? Two parallel studies implicate a peptidylprolyl *cis*/*trans* isomerase (PPIase) (32, 180). PPIases are a recently appreciated class of enzymes that can regulate the

activity of other proteins by modifying the conformation of proline-containing motifs. Remarkably, adding the PPIase inhibitor juglone to cell-free extracts effectively blocks auxin-dependent binding of SCF^{TIR1} to Domain II in several AUX/IAA proteins (32). Another chemical, sirtinol, promotes the degradation of AUX/IAA proteins and accentuates auxin-mediated responses (180). An *Arabidopsis* sirtinol-resistant mutant (*sir1*) was isolated that is auxin hypersensitive, indicating that SIR1 is a repressor of auxin-induced AUX/IAA degradation. Cloning the *SIR1* locus revealed that it encodes a protein related to PPIases (180). However, because SIR1 represses auxin action, it cannot be the PPIase responsible for initiating AUX/IAA protein degradation, but it could reverse the action of another PPIase. The most parsimonious interpretation is that one or more PPIases work coordinately/antagonistically to transmit the auxin signal to a conformational change within Domain II of AUX/IAA proteins; this change then promotes the interaction of AUX/IAA proteins with SCF^{TIR1}, leading to ubiquitination and subsequent degradation of the repressors.

The Ub/26S proteasome pathway is also involved in auxin signaling downstream of the AUX/IAA-SCF^{TIR1} checkpoint. The RING E3 SINAT5 negatively regulates auxin signaling by degrading the transcriptional activator NAC1, which promotes auxin-induced lateral root formation (166). In addition to auxin perception, plants also have sophisticated mechanisms for auxin transport that are used to establish gradients of the hormone necessary for tropic responses. The abundance of the auxin efflux carrier EIR1 is increased in an *axr1-3* background, suggesting that this transporter is a target for a RUB1-regulated SCF E3 (130).

GIBBERELIC ACID (GA) Like those for auxins, early events in GA signaling exploit the inactivation of nuclear-localized repressors. In several plant species, GA treatment decreases the abundance of a family of DELLA transcription factors with the loss blocked by MG132 (49). Recently, related F-Box proteins connected to GA signaling were identified in *Arabidopsis* [SLY1 (98)] and rice [GID2 (118)], based on the GA insensitivity of the corresponding mutants. Their associated SCF E3s promote the degradation of the DELLA proteins, RGA (REPRESSOR OF GA1-3) and SLR1 (SLENDER RICE 1), respectively (98, 118). Rice SCF^{GID2} recognizes SLR1 following SLR1 phosphorylation by a GA-activated kinase (118). In *Arabidopsis*, auxin may also promote DELLA protein breakdown, possibly as a way to integrate the actions of GA and auxin to coordinate plant growth (48). Similarly, phosphorylation-dependent degradation of the BZR1 and BZR2 transcription activators may regulate responses to the brassinosteroid hormones that are structurally related to GAs (68). Here, brassinosteroids appear to block phosphorylation of these factors by the GSK3-like kinase BIN2, thus preventing their Ub/26S proteasome-mediated degradation.

ABSCISIC ACID (ABA) ABA controls many aspects of embryonic and seedling development, including the growth arrest of germinating seedlings in response to

adverse conditions such as drought or salt stress. A key *Arabidopsis* regulator in this postgerminative arrest is ABI5, a bZIP transcription factor whose abundance is increased by ABA at both the transcriptional and post-transcriptional levels (95). ABA increases ABI5 protein levels by inhibiting its ubiquitination and turnover by the 26S proteasome, possibly by changing its phosphorylation status.

At least two proteins play a key role in ABI5 degradation, ABI5-binding protein (AFP) and the 26S proteasome RP subunit RPN10. AFP interacts with ABI5 through its conserved Domain III and acts as a negative regulator of ABI5 abundance, possibly by stimulating its turnover (96). A role for RPN10 was revealed by analysis of the *Arabidopsis rpn10-1* mutation, which removes the C-terminal UIM but does not detectably affect 26S proteasome integrity (132). Like ABI5-overexpression lines, *rpn10-1* seedlings are hypersensitive to ABA and high sugar and salt concentrations. The phenotype is caused by the selective stabilization of ABI5, especially in the presence of ABA (132).

This link between RPN10 and ABA signaling sheds a new light on the reported interaction between RAD23 and VP1, which is the rice ortholog of *Arabidopsis* ABI3 (121). RAD23 was originally identified in yeast as a component of the DNA damage response pathway and is now thought to act synergistically via its UBL domain with RPN10 to deliver substrates to the 26S proteasome (19). Because ABI5 works as a heterodimer with ABI3 to control the transcription of ABA-responsive genes, one testable model is that RAD23 promotes ABI5 proteolysis by linking the ABI3/ABI5 complex to RPN10.

CYTOKININS Although a number of factors in cytokinin signaling have been identified, none reported thus far appear to be components of the Ub-conjugation cascade. However, *rpn12a-1* plants are less sensitive to exogenous cytokinins and [*Erratum] display a characteristic set of phenotypes expected for decreased cytokinin sensitivity and analysis of an *Arabidopsis* mutant affecting the RP subunit RPN12a has provided a link to the 26S proteasome (131). The *rpn12a-1* mutation does not affect the cytokinin-induction of *ARR5*, part of the rapid response pathway for cytokinin, indicating that RPN12a does not work early in the transduction chain (131). An interesting possibility is that RPN12a controls the half-life of one or more cell cycle proteins that act as downstream mediators of cytokinin-induced cell division and development (31).

JASMONIC ACID Like those for auxin and GA, jasmonate signaling has been directly connected to the Ub/26S proteasome pathway through the discovery of an essential F-Box protein COI1 (169). Like TIR1, COI1 assembles with ASK1 and 2, CUL1, and RBX1 to form a SCF^{COI1} complex that is regulated by the RUB1 cycle (30, 43, 169). The phenotype of *coi1* mutants suggests that SCF^{COI1} targets one or more repressors of the jasmonate response. One candidate is the histone deacetylase RPD3b, which interacts with the presumed target-binding LRR of COI1 (30).

*Erratum (14 May 2004): See online log at http://arjournals.annualreviews.org/errata/arplant

ETHYLENE A role for the Ub/26S proteasome in ethylene regulation was proposed earlier based on the short half-life of ACC synthase, the rate-limiting enzyme in ethylene biosynthesis (18, 153). Recent data suggest that turnover of the *Arabidopsis* transcriptional regulator EIN3 is also a critical control point in ethylene signaling (174). EIN3 degradation is dramatically attenuated by ethylene but enhanced by glucose, suggesting that its stability plays a pivotal role in integrating nutritional and hormonal cues. EIN3 ubiquitination involves an SCF complex generated by the incorporation of the LRR-containing F-Box proteins, EBF1 and 2 (EIN3-BINDING F-BOX), that directly bind EIN3 (50a, 60a, 110a). EBF1/2 are most related to yeast Grr1, an F-Box protein required for cell cycle, polarized bud development, and sugar sensing (29). Remarkably, EBF1 and 2 have separate functions in suppressing EIN3 accumulation over a range of ethylene concentrations (50a). EBF1 promotes EIN3 degradation at low ethylene concentrations, whereas EBF2 inhibits excess EIN3 stabilization at saturating ethylene levels. The combination of both functions is essential for plant development since an *ebf1 ebf2* double mutant showed a severe developmental arrest.

Responses to the Abiotic Environment

LIGHT SIGNALING Light is monitored by an array of photoreceptors including the red/far-red light-absorbing phys and the blue/UV-A light absorbing cryptochromes (crys). Both phyA and cry2 are rapidly degraded by the Ub/26S proteasome pathway upon light absorption (21, 127). For phyA, this turnover requires a domain near the chromophore-binding site (21). For cry2, prior phosphorylation of the photoreceptor is essential for recognition (127). Ultimately, a number of downstream transcription factors are stabilized by the light signal, including HY5, HYH, and LAF1. One important factor in this dark-/light-regulated turnover is the RING-E3 COP1 (73, 106, 125). In the dark, COP1 is in the nucleus where it represses photomorphogenesis by degrading factors such as HY5 and HYH in cooperation with the UEV COP10, and a COP1-related RING-E3 CIP8 (63, 137). Both blue and red light remove COP1 from the nucleus, thus relieving this repression. cry1 and 2 directly interact with COP1, which for cry2 appears to mediate its degradation (127). For phys, it is unclear how their light activation is connected to COP1 downregulation.

Two other E3s appear to negatively influence phy-mediated light signaling in *Arabidopsis*. The F-Box protein EID1 acts upstream of COP1 and inhibits phyA-mediated responses (35). EID1 does not influence phyA degradation, indicating that its presumed SCF^{EID1} complex targets one or more factors activated by phyA. SPA1, another suppressor of phyA signaling, directly controls COP1 activity. This WD-40 protein appears to function in a negative feedback loop in phyA signaling by enhancing the ability of COP1 to ubiquitinate the LAF1 and HY5 transcription factors (116a, 125). In contrast, the F-box protein AFR (ATTENUATED FAR-RED RESPONSE) promotes phyA-mediated responses, most likely by targeting a repressor of phyA signaling (63a). Ultimately, the CSN participates in light

perception; in fact, many of the CSN subunits were first identified from genetic screens searching for photomorphogenic mutants (126). Whether this role can be explained solely by the derubinating activity of the CSN is not yet known.

CIRCADIAN RHYTHMS Given that the circadian clocks of bacteria and animals exploit protein turnover for control, it was anticipated that proteolysis would also influence the plant counterparts. This possibility was confirmed by the discovery that ZTL, FKF1, and LKP2 help entrain the clock (102, 133). These proteins harbor an N-terminal LOV domain that can bind flavins, followed by a F-Box motif and C-terminal Kelch repeats. This unique modular organization suggested that ZTL/FKF1/LKP1 are blue-light-absorbing F-Box proteins that identify clock regulators in a light-dependent manner. This was confirmed by the recent demonstrations that FKF1 binds flavin mononucleotide and that its LOV domain can act as a light sensor (76a). Furthermore, ZTL targets the circadian regulatory protein TOC1 (TIMING OF CAB EXPRESSION 1) for breakdown in a dark-dependent manner, suggesting that light changes its target affinity (96a). Because *ztl* and *fkf1* mutants display different phenotypes, it is likely that the resulting SCF E3s recognize nonoverlapping sets of targets. ZTL is itself degraded in a circadian phase-specific manner by the 26S proteasome, which may provide a mechanism for feedback control (81).

ABIOTIC STRESSES Extreme environments often adversely affect proteins by increasing free radicals that encourage denaturation and damage. Removing these proteins by various quality control pathways (ERAD, N-End Rule, the unfolded-protein response, aggresome-mediated degradation) within the Ub/26S proteasome system is critical for cell survival (83, 85, 147). In *Arabidopsis*, the U-Box E3 CHIP may also be important. Its animal orthologs associate with the HSP70 class of chaperones and assist in the ubiquitination and removal of misfolded polypeptides. The mRNA for *AtCHIP* is upregulated by a variety of stresses and overexpression of *AtCHIP* renders *Arabidopsis* seedlings more susceptible to both low- and high-temperature stresses (171). DNA damage is also environmentally induced, and UV is the main culprit. In yeast several Ub/26S proteasome pathway components have been implicated in UV protection by promoting DNA repair and delaying DNA replication until the repairs are complete (70, 159). Many of these (e.g., Ubc2, Rad23) (121; L. Farmer & R.D. Vierstra, unpublished work) have orthologs in plants, suggesting that similar control systems are available. The *Arabidopsis rpn10-1* mutant is hypersensitive to UV- and the DNA-damaging agent mitomycin-C, directly implicating the 26S proteasome (132).

The signal transduction pathways that detect stress also employ Ub/26S proteasome pathway components. For example, cold sensing is negatively regulated by the putative RING-E3 HOS1 (91). Like COP1, its intracellular location is controlled by the environmental signal; during exposure to cold, HOS1 moves from the cytoplasm to the nucleus. Disrupting the RUB1 cycle also affects cold perception, suggesting that an SCF E3 participates as well (123).

Responses to the Biotic Environment

PATHOGENS The Ub/26S proteasome pathway affects pathogen defenses at multiple levels by influencing primary infection, pathogen spread, and defense signaling pathways. The importance of the pathway was first suggested by the discovery that expression in tobacco of Arg48 Ub, which cannot assemble Lys48-linked polyUb chains, accentuates the hypersensitive response, a localized programmed cell death (PCD) that helps limit pathogen spread (9). Subsequently, it was found that treating plants with pathogen elicitors induces the expression of specific Ub/26S proteasome pathway genes, including an E2 (*Os*UBC5b) and a RING E3 (EL5) in rice (138) and several CP subunits of the tobacco 26S proteasome (25). A more direct role was implied by the ability of pathogen invasion to dramatically decrease the half-life of the *Arabidopsis* resistance protein (R) RPM1 (11), a member of a superfamily of presumed pathogen-specific receptors. One protein required for R-mediated defense in barley, RAR1, interacts with the SCF E3-activator SGT1 (2, 3). Because loss of *SGT1* function attenuates the early defense against a wide range of pathogens, this protein likely participates in a convergent reaction directed by various R proteins (107). Presumably, SGT1 helps assemble specific SCF-E3(s) required for many R gene-mediated defense responses.

The defense response at the site of pathogen invasion is normally followed by a more general nonspecific response throughout the plant, ultimately enhancing the expression of pathogenesis resistance proteins (PRs) that help counter future attacks. This systemic response is mediated by hormones such as salicylic acid, ethylene, and jasmonic acid. For jasmonate signaling, the SCF^{COI1} is important and thus jasmonate-resistant *coi1* plants are also more susceptible to pathogen invasion (140). *Arabidopsis* NIM1 (or NPR1) is essential for the signaling cascade that induces PR protein expression. In a screen for *nim1-1* suppressors, the F-Box protein SON1 that may initiate the degradation of systemic resistance activator(s) was identified (79).

Several pathogens have co-opted Ub/26S proteasome pathway components. As examples, the tobacco mosaic virus incorporates a ubiquitinated form of the coat protein into the virion (67), and *Agrobacterium tumefaciens* synthesizes the VirF F-Box protein that is shuttled into the host during infection (119). The pathway may also control the movement of plant virions between cells through the plasmodesmata. For example, the abundance of the tobacco mosaic virus-encoded movement protein rises *in planta* in response to MG132, suggesting that it is a 26S proteasome target (114).

SELF-INCOMPATIBILITY Many plant species avoid inbreeding by using mechanisms that differentiate self from nonself pollen and then prevent self-pollen germination and/or growth (103). Pollen/pistil interactions are controlled by a highly polymorphic S locus that contains allelic versions of a male- and female-specific pair of factors. For instance, in *Brassica napus*, the female S-component is a family of SRK receptor kinases expressed at the stigma surface that have allelic

specificity for a family of SCR/SP11 male ligands expressed in the pollen coat. SCR/SP11 binding to its cognate SRK activates the receptor, eventually inducing pollen rejection. A role for the Ub/26S proteasome pathway was demonstrated with the identification of ARC1, an SRK-interacting protein that participates in the self-incompatibility reaction. ARC1 belongs to the U-Box E3 family and can function as a Ub ligase in vitro (135). Loss of ARC1 substantially diminishes the pool of ubiquitinated proteins within the pistil during an incompatible interaction, suggesting that ARC1 has many targets.

E3s may also be involved in the self-incompatibility reactions of *Rosaceae*, *Solanaceae*, and *Scrophulariaceae*, which use a pistil-expressed S-RNase as the female determinant to degrade RNA in the pollen tube of self pollen. In almond, the S locus also contains a polymorphic gene encoding a F-Box protein that is pollen specific and thus may function as the male S-component (143). It is tempting to speculate that this F-Box protein maintains pollen viability and thus allows fertilization by promoting the turnover of compatible S-RNases that enter the pollen tube.

Plant Development

FLOWER DEVELOPMENT Previously, the timing and distribution of various regulators in the floral meristem suggested that flowering is controlled, in part, by post-transcriptional mechanisms. This was confirmed by the discovery that *Arabidopsis* UFO (UNUSUAL FLORAL ORGANS) and its *Antirrhinum majus* ortholog FIM (FIMBRIATA), which are important for the function of B-class genes that control petal and stamen development, are F-Box proteins (117, 179). The phenotypes of *ufo* and *fim* mutants imply that the corresponding SCF E3s degrade suppressors of B-function genes, possibly including *Antirrhinum* CHO and DESP and their *Arabidopsis* orthologs (160).

TRICHOME MORPHOGENESIS The initiation and morphogenesis of trichomes depend on the concerted action of a large number of regulatory proteins that promote or repress various stages in the development of these polyploid epidermal cells. Recently, it was discovered that the *Arabidopsis* HECT-E3 UPL3 is involved in branch initiation and endoreplication (39). Loss of UPL3 activity increases both trichome branching and nuclear DNA content. The phenotype implies that the target(s) of UPL3 are activator(s) that must be removed toward the end of trichome differentiation to prevent excessive branching and DNA replication. Potential targets include the GL1/TTG1/GL3 transcription regulatory complex, which is a key effector of trichome development (39).

SENESCENCE Given the massive amount of protein recycling that occurs during senescence, especially in leaves, it was proposed early on that the Ub/26S proteasome is involved (152). Recently, several senescence mutants provided a direct connection to senescence signaling. The *ORE9* gene, identified from a screen for

delayed senescence mutants, encodes a LRR-containing F-Box protein that assembles with ASK1, presumably to form a SCF^{ORE9} complex (163). *ORE9* was also identified by the shoot-branching mutant *max2-1*, suggesting that this SCF E3 has additional roles/targets outside senescence (134).

Some senescent processes in plants are reminiscent of animal PCD. In *Drosophila*, the Ub/26S proteasome system has been implicated in PCD via its role in degrading DIAP1 (147). DIAP1 is a member of an IAP1 family of PCD inhibitors that binds to and neutralize caspases, and removes factors that enhance PCD by its RING E3 Ub-ligase activity (36). Caspases then stimulate DIAP1 turnover by removing a short N-terminal region, thus promoting breakdown of the truncated DIAP1 (now bearing an N-terminal asparagine) by the N-End Rule pathway. Recognition of truncated DIAPI using the N-End Rule pathway first requires N-terminal deamidation by an asparagine deamidase and subsequent N-terminal arginylation by an arginyl-tRNA:protein arginyltransferase (R-transferase) (147). It was previously established that the N-End Rule pathway is active in plants (6, 164). Recently, the N-End Rule was connected to plant senescence by the discovery that *dls1*, an *Arabidopsis* delayed senescence mutant, disrupts expression of a R-transferase that can support the N-End Rule pathway (177). Although a plant ortholog of DIAP1 has not yet been found, tobacco can respond to nonplant IAP1 activities, which suggests that one exists (34). Taken together, plants like metazoans may exploit the N-End Rule pathway to regulate PCD by degrading IAP1-like proteins. Plant PCD also appears to involve the 26S proteasome (80). For example, tracheid differentiation requires the 26S proteasome among other proteolytic activities during the maturation phase to generate this dead water-conducting element (162).

OTHER ROLES OF UBIQUITINATION AND THE 26S PROTEASOME

In addition to their more traditional roles, data from yeast and animals indicate that components of Ub/26S proteasome pathway can also have other functions, some of which are likely used by plants. Many of these arise from the ability to attach a single Ub or assemble polyUb chains using lysines other than Lys48. A number of monoubiquitinated proteins have been identified, including the H2A and H2B subunits of the core nucleosome (5) and numerous receptors and transporters at the plasma membrane (71). For the histones, adding a single Ub can promote or silence genes, possibly by affecting the methylation of histone H3. Monoubiquitination of the membrane-bound receptors/transporters does not trigger their degradation by the 26S proteasome but instead shuttles them via an endosome-mediated trafficking pathway to the lysosome/vacuole for breakdown (71). Conversely, some newly synthesized proteins also use monoubiquitination to direct transport from endosomes to the plasma membrane. Several mammalian viruses commandeer this trafficking to bud from cells, raising the possibility that some plant viruses do

the same. An important intermediate in this trafficking is the multivesicular body (MVB) (71). MVB assembly and function require several UIM domain-containing proteins (e.g., yeast Vsp27 and Epsin) that help identify monoubiquitinated cargo and deliver it to the appropriate destination.

For those proteins modified with polyUb chains, linkages involving lysines besides Lys48 are evident. One common chain involving Lys63 linkages is not used as a 26S proteasome targeting signal but plays an important role in postreplicative DNA repair and other functions (5, 110, 159). For example, yeast expressing an Arg63 Ub mutant, which cannot assemble Lys63-linked chains, is hypersensitive to DNA-damaging agents. In yeast, two RING E3s, Rad5 and Rad18, and the Ubc2/Ubc13 E2/UEV dimer are responsible for adding Lys63-linked chains to the DNA-repair protein Proliferating Cell Nuclear Antigen (PCNA) (5, 145). In other examples, the yeast ribosomal subunit L28 and the mammalian IκB kinase that targets IκB for ubiquitination by phosphorylation are modified with Lys63-linked polyUb chains.

In addition to completely degrading ubiquitinated proteins, there is evidence that the 26S proteasome only partially degrades some substrates. Remarkably, the released fragments are bioactive. Examples include human Nuclear Factor-κB (NF-κB) and yeast Spt23 and Mga2 (75). These transcription factors are inactive in their full-length forms but become active upon ubiquitination and partial processing by the 26S proteasome. Whether a similar level of regulation exists in plants is unknown. Recently, it was discovered that the RP RPT ring, independent of the rest of the 26S proteasome, associates with transcription complexes (5). It has been proposed that the AAA-ATPase activity of this ring is recruited during transcription to help remove chromatin organizing proteins and repressors to enhance access to promoters. In a similar vein, the RP without the CP was found to have a nonproteolytic role in nucleotide-excision DNA repair (5).

CONCLUSIONS AND PERSPECTIVES

Since its discovery in plants nearly 20 years ago (152), there has been remarkable progress in defining the organization and functions of the Ub/26S proteasome pathway. Particularly insightful have been recent bioinformatic analyses of the *Arabidopsis* genome, the use of 26S proteasome inhibitors, and the expanding array of pathway mutants, particularly in *Arabidopsis*. Now that many of the core components are known, rapid progress is expected in defining the roles of the Ub/26S proteasome pathway in plant growth, development, and environmental adaptation. Presently, many important questions remain unanswered. For example, how are E3s regulated? How are polyUb chains assembled? How does the cell know which proteins should be monoubiquitinated or modified with polyUb chains of various linkages? Why have plants expanded their dependence on the Ub/26S proteasome pathway compared to their animal brethren? What are the functions of the various subunits of the 26S proteasome RP, and do the multiple proteasomes in plants

have distinct functions? Finally, what are the substrates of the Ub/26S proteasome pathway in plants? Even with the bevy of events controlled by ubiquitination, only a handful of targets have been identified, with an even smaller number actually demonstrated to be ubiquitinated in planta.

The question of target identification may be one of the most difficult to answer for two reasons. First, from the analysis of known ubiquitination targets, it appears that many different recognition motifs exist and that these motifs often are post-translationally modified (108, 110, 159). Consequently, it is unlikely that sequence comparisons of short-lived proteins will be broadly helpful. Second, Ub conjugates are by nature heterogeneous, transient, and present at extremely low levels, making their chemical analysis difficult. Hopefully, the application of mass spectrometry coupled with the use of tagged versions of Ub, which has recently proven successful for yeast (108), may help us define the plant "ubiquitinome," i.e., the subset of intracellular proteins that are ubiquitinated.

Ultimately, dissecting the Ub/26S proteasome pathway should reveal how protein turnover controls plant biology. However, these discoveries may only represent the tip of the iceberg. Genomic analyses of *Arabidopsis* reveal the presence of other proteolytic systems and a large cache of proteases (e.g., 37). Through their combined action, plants maintain and regulate a very active protein cycle necessary for proper growth, development, and homeostasis.

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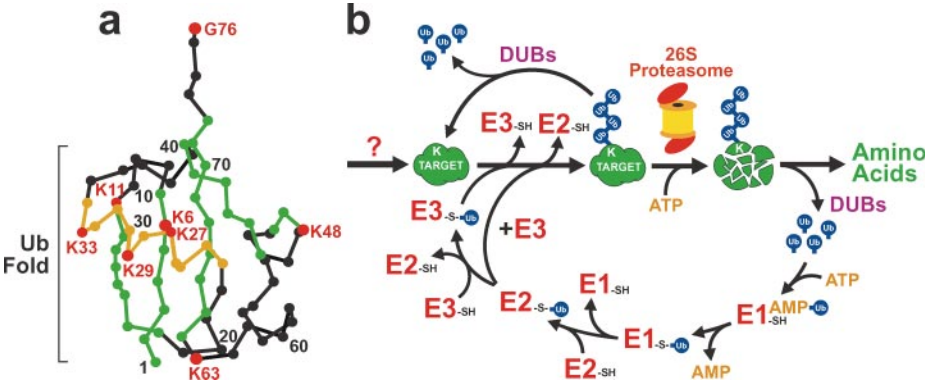

Figure 1 Ub and the Ub/26S proteasome pathway. (*a*) The three-dimensional structure of plant Ub. The Ub fold is indicated with its mixed β sheets and α helix shown in green and orange, respectively. The lysines (K) at positions 6, 11, 27, 29, 48, and 63 that can participate in forming polyUb chains and the C-terminal glycine that forms the isopeptide bonds with targets are indicated in red. (*b*) Diagram of the Ub/26S proteasome pathway. The pathway begins with the adenosine triphosphate (ATP)-dependent activation of Ub by E1, followed by transfer of the Ub to an E2, and finally attachment of the Ub to a lysine in the target protein with the help of an E3. Once the Ub-protein conjugate is formed bearing a polyUb chain, it is either recognized by the 26S proteasome and degraded in an ATP-dependent process with the concomitant release of Ub monomers or the conjugate is disassembled by deubiquitinating enzymes (DUBs) to regenerate both the target protein and Ub intact.

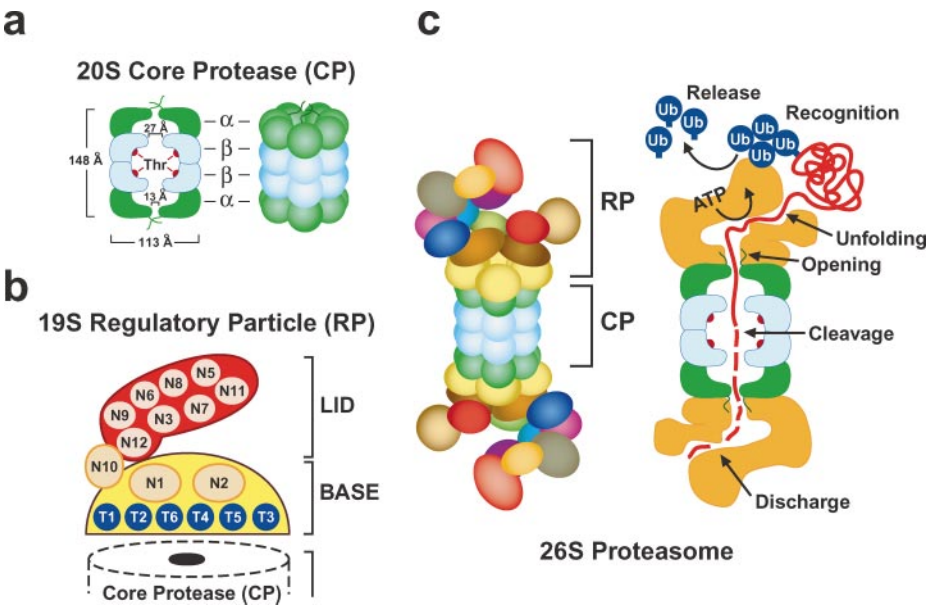

Figure 2 Organization and structure of the 26S proteasome. (*a*) Organization of the 20S core protease (CP) based on the crystal structure of the yeast particle (60). The positions of the active-site threonines are shown. (*b*) Predicted organization of the 19S regulatory particle (RP) based on its subunit interaction map with the Lid and Base shown in red and yellow, respectively (46). The RP AAA-ATPase (RPT) subunits are shown in blue. The RP non-ATPase subunits are shown in orange. (*c*) Diagram of the 26S proteasome combined with the predicted activities of the complex during the degradation of ubiquitinated proteins. Adapted from Reference 154.

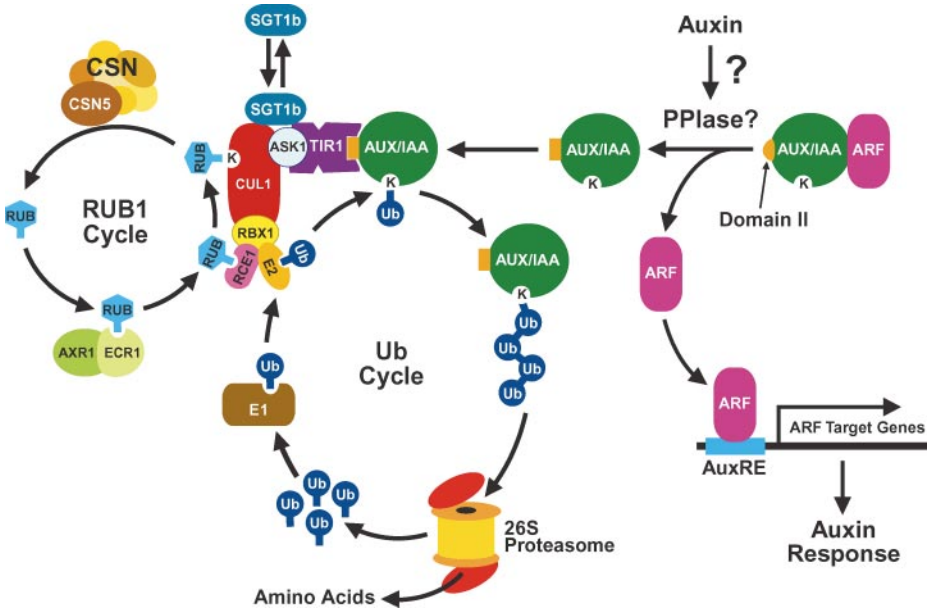

Figure 3 Model for the auxin-dependent degradation of AUX/IAA proteins by the Ub/26S proteasome pathway. The process begins with a peptidylprolyl *cis/trans* isomerase (PPIase) altering the conformation of Domain II in AUX/IAA proteins either before or after their dissociation from ARFs. The AUX/IAA proteins are recognized and ubiquitinated by a Ub-conjugation cycle involving an E1, an E2, and the SCF^{TIR1} E3, which consists of a Cullin-CUL1, RBX1, the SKP1-ASK1, and the F-Box protein TIR1. K denotes the lysine in AUX/IAA proteins that bind Ub. The resulting polyUb AUX/IAA conjugates are degraded by the 26S proteasome with the release of the Ubs. The activity of SCF^{TIR1} is modulated by reversible attachment of RUB1 to the CUL1 subunit using a RUB1 conjugation cycle involving a heterodimeric E1 (consisting of AXR1 and ECR1) and an E2 (RCE1) and the derubinating activity of CSN subunit CSN5. The SCF^{TIR1} E3 may also be regulated by association with SGT1b. Free ARFs then activate/repress the expression of a number of auxin-regulated genes by binding to their auxin-responsive elements (Aux-RE). Adapted from Reference 69.

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