The Cullin-RING Ubiquitin-Protein Ligases

Zhihua Hua and Richard D. Vierstra

Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706-1574;
email: hua3@wisc.edu, vierstra@wisc.edu

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Abstract

The posttranslational addition of ubiquitin (Ub) helps control the half-life, localization, and action of many intracellular plant proteins. A primary function is the degradation of ubiquitylated proteins by the 26S proteasome, which in turn plays important housekeeping and regulatory roles by removing aberrant polypeptides and various normal short-lived regulators. Strikingly, both genetic and genomic studies reveal that Ub conjugation is extraordinarily complex in plants, with more than 1500 Ub-protein ligases (or E3s) possible that could direct the final transfer of the Ub moiety to an equally large number of targets. The cullin-RING ligases (CRLs) are a highly polymorphic E3 collection composed of a cullin backbone onto which binds carriers of activated Ub and a diverse assortment of adaptors that recruit appropriate substrates for ubiquitylation. Here, we review our current understanding of the organization and structure of CRLs in plants and their dynamics, substrates, potential functions, and evolution. The importance of CRLs is exemplified by their ability to serve as sensors of hormones and light; their essential participation in various signaling pathways; their control of the cell cycle, transcription, the stress response, self-incompatibility, and pathogen defense; and their dramatically divergent evolutionary histories in many plant lineages. Given both their organizational complexities and their critical influences, CRLs likely impact most, if not all, aspects of plant biology.

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Ubiquitin (Ub): a 76-amino acid protein that becomes covalently attached to other proteins

26S proteasome: a 2.5-MDa complex that degrades proteins modified with Ub

Ub/26S proteasome system (UPS): pathway for degrading proteins via the 26S proteasome that first involves the selective attachment of Ub

INTRODUCTION TO UBIQUITIN AND THE UPS

Life is exquisitely birthed, maintained, and reshaped by the synthesis of new proteins, their subsequent assembly into functional structures, and their final removal by various proteolytic routes. Whereas the machineries directing protein synthesis and assembly are relatively well understood, we are only beginning to appreciate the underpinning mechanisms responsible for the final catabolic step in a protein's life and, in particular, the roles that various posttranslational modifiers play in this process. In fact, it is now clear that plants and animals employ a collection of small polypeptide modifiers that affect protein breakdown (42, 86). The founding member of this family—ubiquitin (Ub)—was first identified during the Nobel Prize–winning research of Hershko and colleagues investigating intracellular proteolysis (83). Upon addition, Ub serves as a reusable tag that selectively commits proteins for destruction as well as other nonproteolytic outcomes. Subsequently, a number of functionally distinct "Ub-fold" proteins have been discovered, including related to Ub (RUB)-1/NEURAL-PRECURSOR-CELLS DEVELOPMENTAL-DOWNREGULATED (Nedd)-8, membrane-anchored Ub-fold protein (41a), autophagy-8 and -12, and small-Ub-like-modifier (42, 86). Surprisingly, these relatives can work either alone, together, or in conflict with Ub in regulating protein turnover, thus highlighting the depth and breadth of posttranslational events that affect a protein's life span.

As the name implies, Ub is a highly conserved, small polypeptide ubiquitously distributed among eukaryotes. Its ligation is accomplished by an ATP-dependent reaction cascade involving the sequential action of Ub-activating (E1s), Ub-conjugating (E2s), and Ub-protein ligase (E3s) enzymes (**Figure 1*a***) (181, 212). The final step links, via an isopeptide bond, the C-terminal glycine carboxyl group of Ub to a free lysl ε-amino group in the target. In some cases, only a single Ub is attached (mono-ubiquitylation). More often, reiterative rounds of conjugation assemble a Ub polymer onto the target (poly-ubiquitylation), using one or more lysines from previously attached Ubs for concatenation (**Figure 1*b***). The number and arrangement of the linked Ubs then imbue important structural information that expands the functionality of the Ub moiety (147). Ubiquitylated proteins have several possible fates. Substrates modified with poly-Ub chains, especially those linked via Lys-11 or Lys-48, are degraded by the 26S proteasome, a 2.5-MDa proteolytic complex that breaks down the substrate but releases the attached Ubs intact for reuse. Through this Ub/26S proteasome system (UPS), plants and animals selectively

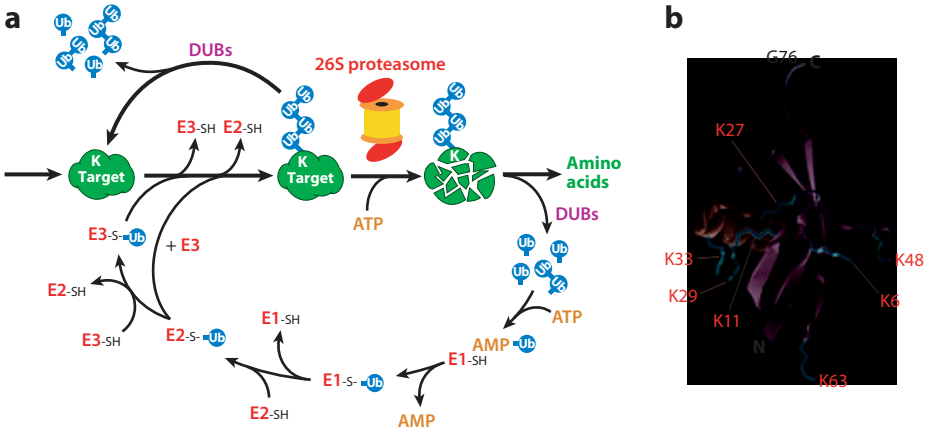

Figure 1

Ubiquitin (Ub) and the Ub-26S proteasome system (UPS). (*a*) Diagram of the UPS. The pathway begins with adenosine triphosphate (ATP)-dependent activation of Ub by an E1, followed by transfer of the activated Ub to an E2, and then final attachment of the activated Ub to the target with the help of an E3. The resulting product is a Ub-protein conjugate where the C-terminal Gly carboxyl group of Ub is linked through an isopeptide bond to an accessible amino group (typically lysine ε-amino) in the target or another Ub if poly-Ub chains are added. After iterative assembly, the Ub-protein conjugate can be disassembled by deubiquitylating enzymes (DUBs) to release the target protein and Ubs intact or the target can be broken down by the 26S proteasome, with the concomitant release of the bound Ub molecules by DUBs. (*b*) Three-dimensional ribbon model of plant Ub (204). The side chains from the seven lysines in Ub that can be used for poly-Ub chain formation are shown in red. The β strands are in green, the α helices are in cyan, and the C-terminal Gly76 used to ligate Ub to other proteins is indicated. N, N terminus; C, C terminus.

remove misfolded or mutant polypeptides and the majority of normal intracellular regulators in the cytoplasm and nucleus (181). Alternatively, mono-ubiquitylated and Lys-63 poly-ubiquitylated targets bound to the plasma membrane are often internalized and transported to the vacuole/lysosome for breakdown. Ub addition can also be reversed by a family of deubiquitylating enzymes (DUBs) that cleave just the isopeptide bond to release both polypeptides intact. This ubiquitylation/deubiquitylation cycle helps direct a number of nonproteolytic events involved in chromatin structure, transcription, and vesicle trafficking (132).

Key to the specificity of ubiquitylation and the arrangement of the bound Ubs are the E3s (181, 212). They recruit substrates, position them for optimal transfer of the Ub moiety from an associated E2, and then stimulate conjugation. Not surprising considering the range of target proteins affected by Ub addition, eukaryotes employ an extraordinarily large collection of E3s to potentially modify an equally large set of targets. For example, comprehensive genome analyses of *Arabidopsis thaliana* predict that it can synthesize more than 1,500 different E3s (43, 60, 64, 91, 118, 131, 188), potentially making this enzyme collection one of the largest in the plant kingdom (202).

One prominent collection of E3s is the cullin-RING ligases (CRLs). They are defined by a common backbone consisting of one of several cullin (CUL) isoforms, the really interesting new gene domain (RING)-containing protein RING-BOX (RBX)-1 (Roc1/Hrdt in animals), and a variety of adaptors that recognize and deliver appropriate substrates for ubiquitylation (14, 145). In the past decade primarily using *Arabidopsis* as the model, researchers have shown that CRLs are central to numerous processes in plants, including hormone and light perception where some even act as receptors, regulation of the cell cycle and transcription, self-recognition, and the

Cullin-RING ligases (CRLs): types of Ub ligases that share the cullin scaffold along with the RBX subunit and one of a number of target recognition modules

Cullin (CUL): arch-shaped protein that provides the scaffold for CRLs

RBX1: RING-containing subunit of CRL complexes that docks E2s carrying activated Ub

RUB1/Nedd8: a Ub-like protein that stimulates the ubiquitylation activity of CRLs upon covalent attachment to the cullin subunit

response to biotic and abiotic challenges (115, 203). The purpose of this review is to update our current understanding of CRLs in plants with respect to their structural organization, regulation, functions, and complicated evolutionary histories. Wherever possible, we emphasize data from plant systems but include results from other organisms to fill in knowledge gaps. Hopefully, it becomes apparent that the CRLs can influence much of plant biology. The reader is referred to additional reviews on the plant UPS, which describe the 26S proteasome, DUBs, other important E3 types, and various regulatory factors (44, 87, 164, 181, 203, 211).

ORGANIZATION AND STRUCTURE OF CRLS

All CRLs share a common molecular architecture, which has been modified to presumably expand the repertoire of substrates, diverse modes of regulation, and possibly unique ways to attach Ub moieties. Their composite structures are reasonably well known thanks in part to several representative near-complete or partial three-dimensional models (e.g., 3, 62, 67, 78, 121, 128, 193, 215, 233, 235), including two from plants (174, 192). Using motifs that distinguish each of the subunits, it is now relatively easy to identify potential CRL orthologs in any plant species by bioinformatic methods.

The signature subunit is the CUL scaffold protein. Plants synthesize three main CUL types (CUL1/CU2a/b, CUL3a/b, and CUL4 in *Arabidopsis*) (64, 175), each of which assembles a distinct CRL collection. As illustrated by the structure of a human CRL designed to ubiquitylate the phosphorylated cell-cycle regulator p27 (233), CULs employ a string of three-helix bundles to generate an elongated shallow arc (**Figure 2*a***). The C-terminal region downstream of the bundles contains two helical domains that create a V-shaped cleft; this cleft becomes tightly occupied by the N-terminal helix of RBX1 to create a stable CUL/RBX1 catalytic core. The C-terminal 70-amino-acid RING-H2 domain in RBX1 provides a docking platform for E2s charged with Ub. This RING domain is formed by an octet of cysteines and histidines in a C3/H2/C3 arrangement, which chelates two zinc atoms; the resulting fold creates a shallow pocket. Binding of the E2-Ub to this pocket allosterically promotes Ub transfer from the E2 directly to the substrate. CRLs in yeast and animals use members of the Cdc34 E2 family as the Ub shuttles, which are distinguished by a long C-terminal acidic extension that helps tether the E2 to the CUL (107). It remains unclear which E2 type(s) among the 14 or so present in plants (111) is its functional counterpart(s), in part because a clear sequence ortholog of Cdc34 is not obvious in plants. Adjacent to the RBX1-binding site in CULs is a positionally conserved lysine that becomes reversibly modified with RUB1/Nedd8 during the ubiquitylation cycle (see below).

The N-terminal bundle of each CUL terminates in a hydrophobic/polar patch that binds specific sets of substrate adaptors (**Figure 2*a–c***). These adaptors can best be described as baseball catcher's mitts [e.g., the β propeller of kelch and WD-40 domain repeats, and the solenoid shape of leucine-rich repeats (LRRs) (121, 140, 174, 192, 215)], which create a large pocket to grasp the substrate. When a holo-complex is assembled with its substrate, a C-shaped quaternary structure that correctly positions the substrate next to the E2-Ub intermediate is formed (**Figure 2*b***). Presumably, the substrate is oriented in such a way as to bring one or more accessible lysines close to the activated Ub. Given the ample space predicted between bound substrates and the docked E2-Ub moiety (~60 Å) (193, 215), the exploitation of adaptors with widely varied shapes for substrate selection, and the fact that ubiquitylation can sometimes be promiscuous with respect to the substrate lysines, it is likely that the substrate is bound to the CRL with considerable wobble. Consequently, simply positioning one or more accessible substrate lysines in a "hot zone" close to the E2 may be sufficient to drive Ub transfer (17).

Once bound to the CRL, the substrate is ubiquitylated with at least four Ub moieties needed to send the substrate to the 26S

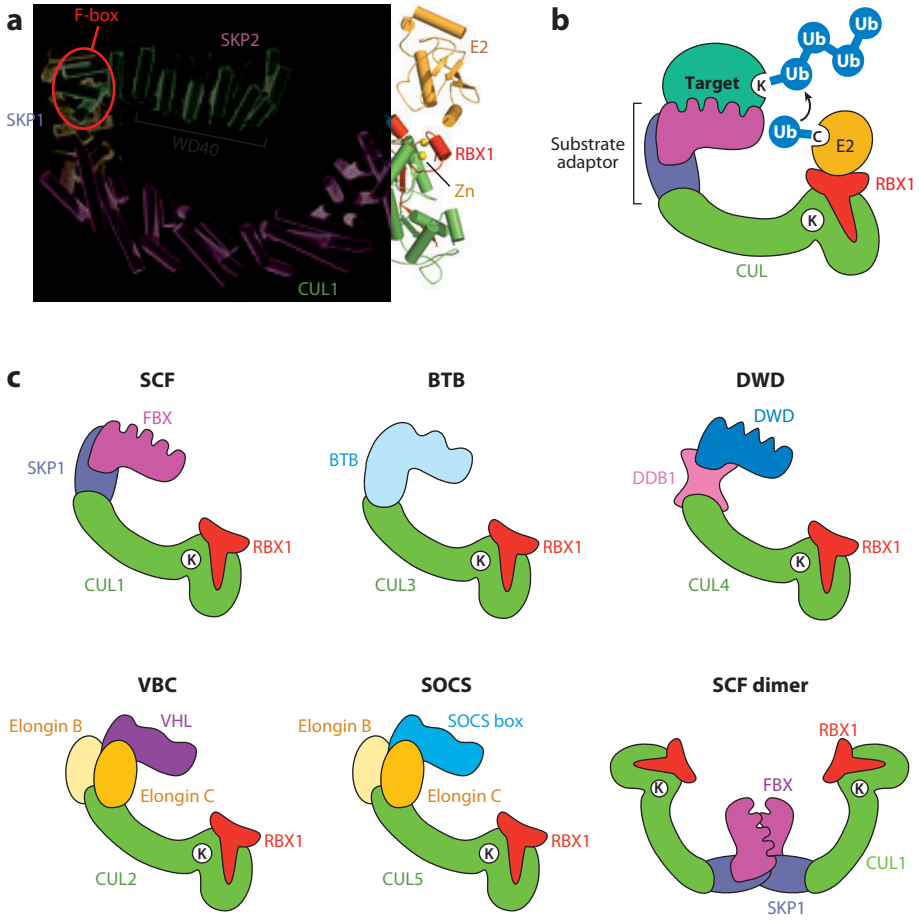

Figure 2

Three-dimensional structures and subunit organization of cullin-RING ligases (CRLs). (*a*) Three-dimensional ribbon model of the human SCF^{SKP2} complex. The F-box domain and the propeller WD-40 repeat domains in SKP2 are indicated. The yellow spheres in RBX1 indicate the locations of the chelated zinc atoms (adapted from Reference 233). (*b*) Diagram of a CRL with its target based on the three-dimensional structure of several representatives. The various substrate adaptor configurations are shown in panel c. The K in the target and the CUL (cullin) are the acceptor sites for ubiquitin (Ub) and RUB1/Nedd8, respectively. The C in the E2 locates the active-site cysteine that binds activated Ub. The CRL shown ultimately assembles a chain of multiple Ubs. (*c*) Organization of CRL complexes found in plants and metazoans. Diagrams are for SCF, VHL, BTB, DWD, and SOCS box-type CRLs, which are assembled with the CUL1, 2, 3, 4, and 5 isoforms, respectively. Only the SCF, BTB, and DWD complexes have been detected in plants to date. The lower right cartoon shows the predicted dimeric structure for a SCF CRL complex based on the analysis of the yeast SCF^{Cdc4} dimer (adapted from Reference 193).

proteasome (147). From the analysis of in vitro reactions using purified components, Ub addition appears processive (107, 148). In vivo, both processive and direct addition of preassembled chains are likely, given the substantial amount of free poly-Ub chains that exist inside plant and animal cells (200). Inhibitor studies using mammalian cells suggest that at least 20% of all proteasome-mediated breakdown is CRL dependent (183). In accord, RBX1 and each of the main CUL types in *Arabidopsis* are essential; null homozygous mutants display early embryo

SCF: CRL that contains SKP1, CUL1, RBX1, and an FBX protein as the target recognition subunit

BTB: CRL that contains CUL3, RBX1, and a broad complex/tramtrack/bric-a-brac protein as the target recognition subunit

DWD: CRL that contains DDB1, CUL4, RBX1, and a DWD protein as the target recognition subunit

F-Box (FBX): ~40-amino-acid domain that helps dock FBX proteins with SKP1

arrest (54, 64, 175) while weak alleles exhibit a range of pleiotropic defects (9, 19, 72, 116, 118, 159).

Thus far, five major types of CRLs have been identified in metazoans, which are easily distinguished by both the nature of their substrate adaptors and by their associated CUL (14, 145). Only three of these, SCF, BTB, and DWD complexes, have been detected thus far in plants (**Figure 2*c***).

SCF CRLs

The SCF CRLs are so named after their founding member, which consists of the CUL1 isoform (Cdc53 in yeast) and a substrate adaptor composed of the S-phase kinase-associated protein (SKP)-1/cyclin-F heterodimer (52, 180). It was discovered subsequently that cyclin-F is part of a large protein family that is distinguished by a common N-terminal ~40-amino-acid domain called the F-box (FBX) (6). As revealed by the three-dimensional structure of several SCF complexes (see **Figure 2*a***), this reasonably degenerate FBX sequence assumes a compact trihelical fold that forms an interlocked interface with a broad shallow pocket in SKP1 (169, 215, 233). The SKP1/FBX adaptor docks with the N terminus of CUL1. The primary interface is provided by the ~120-amino-acid BTB/POZ fold in SKP1 with several amino acids in the FBX domain also contacting CUL1 (168, 233). The FBX proteins individually exploit one of a number of C-terminal interaction domains to recruit appropriate substrates (60, 179). By binding SKP1 via the FBX domain and SKP1 then binding CUL1 via its BTB/POZ domain, FBX proteins deliver targets to the CUL1/RBX1 core for ubiquitylation (**Figure 2*b,c***).

In contrast to most animals and yeast, which encode one or a few SKP1 proteins and a small set of FBX proteins [e.g., 14, 27, and 69 *FBX* genes in yeast (*Saccharomyces cerevisiae*), *Drosophila melanogaster*, and humans, respectively (179)], plants can assemble a surprisingly diverse array of SCF complexes using large gene families encoding the SKP1 and FBX subunits (**Table 1**). In *Arabidopsis* for example, almost 700 functional *FBX* genes exist that account for almost 2.3% of the protein coding genes along with ~200 pseudogenes

Table 1 Number comparison of cullin-RING ligase adaptors among selected eukaryotes

	Number of CRL adaptors[a]					
	FBX		BTB		DWD	
Species	Number	Reference	Number	Reference	Number	Reference
Plants						
Arabidopsis lyrata	1350/980	(91)				
Arabidopsis thaliana	897/698	(91)	80	(64)	85	(118)
Brachypodium distachyon	998/686	(91)	166	(95)		
Carica papaya	198/154	(91)				
Chlamydomonas reinhardtii	88/83	(91)				
Medicago truncatula	1148/908	(91)				
Oryza sativa	971/764	(91)	192/149	(65)	78	(118)
Physcomitrella patens	258/241	(91)				
Nonplants						
Saccharomyces cerevisiae	20	(179)	5	(187)	20	(81)
Caenorhabditis elegans	~520	(196)	178	(187)	36	(81)
Drosophila melanogaster	27	(179)	85	(187)	75	(81)
Homo sapiens	69	(179)	183	(187)	90	(81)

[a]First number indicates total gene number and second number indicates likely intact genes without predicted pseudogenes.

(60, 91). An assortment of interaction motifs are downstream of the FBX domain, including LRRs, kelch repeats, Tubby, lectin-like, and light-oxygen-voltage (LOV), which can recognize proteins, small molecules, glycosyl moieties, and even flavin chromophores (60, 91, 217, 221). Combined with 19 *SKP1* genes (*ASK1–19*) (51, 60), *Arabidopsis* could theoretically assemble thousands of different SCF complexes. Not surprisingly, individual FBX proteins and their cognate SCF complexes have been connected to almost all facets of plant physiology and development (**Table 2**), including several novel forms that work as hormone (174, 192) and light receptors (106). Furthermore, because many SCF targets in animals and yeast are phosphorylated before Ub transfer (145, 194), it is conceivable that the raison d'être of many plant protein kinases is to control ubiquitylation by SCF CRLs.

BTB CRLs

Following the identification of SCF E3s, a second related CRL complex that includes the CUL3 isoform, RBX1, and members of the broad complex/tramtrack/bric-a-brac (BTB) family was discovered (58, 149, 218). Structurally, BTB proteins act as SKP1/FBX amalgams; accordingly, they contain a BTB/POZ fold related to that in SKP1 to bind CUL3 (235) (**Figure 2*c***). N- or C-terminal to the BTB domain are a variety of protein-protein interaction domains that recognize targets. Interaction domains used by plants include armadillo and ankryin repeats, nonphototropic-hypocotyl (NPH), meprin-and-TRAF-homology (MATH), and tetratricopeptide as well as a variety of novel sequence motifs (64, 65).

Similar to FBX proteins, some eukaryotes express large families of BTB adaptors that can assemble with the CUL3/RBX1 catalytic core (187). For example, the yeast, *Drosophila*, and human genomes encode 5, 85, and 183 BTB proteins, respectively (**Table 1**). *Arabidopsis* and rice (*Oryza sativa*) can assemble 80 and 149 different BTB complexes, respectively, which cluster into ~16 clades depending on the nature of the BTB adaptor (54, 64, 65, 210). Preliminary genetic analyses have linked individual members to various processes ranging from blue- and red-light perception (143; M.J. Christians, D.J. Gingerich, R.D. Vierstra, unpublished), to ethylene biosynthesis (23, 208) and salicylic acid (SA)-mediated defense signaling (190) (**Table 2**).

DWD CRLs

The most recent additions to the CRL pantheon are the DWD CRLs (9, 19, 84). They employ the CUL4 isoform and RBX1, along with a substrate adaptor module consisting of DNA damage-binding protein (DDB)-1 and a set of DDB1-binding/WD-40 domain-containing (DWD) proteins (**Figure 2*c***). As with SKP1 in SCF CRLs, DWD complexes use DDB1 to tether various DWD proteins to the CUL4/RBX1 catalytic core. However, instead of containing a BTB/POZ-like fold to bind CUL4, X-ray crystallographic studies of a human CUL4/RBX1/DDB1 subcomplex revealed that DDB1 has two β-propeller structures: one that connects DDB1 to the N terminus of CUL4 and another that connects the various DWD proteins to DDB1 (3). The consensus 16-amino-acid DWD box within the WD-40 domain of DWD proteins contains a core WDXR motif, which generates the docking site for DDB1 (81).

Using this DWD motif consensus as a query, researchers identified 85 *Arabidopsis* and 78 rice DWD proteins; paired interaction studies have confirmed the association of many of the *Arabidopsis* versions with DDB1 (118). Most metazoans encode comparable numbers, whereas yeast encodes considerably less (81) (**Table 1**). Either N- or C-terminal to the DWD motif of some DWD proteins are various protein interaction domains that presumably provide docking sites for substrates (118). Conversely, others apparently consist solely of the DWD-containing WD-40 domain, implying that additional factors are sometimes required for substrate

Table 2 Functionally characterized cullin-RING ligase substrate adaptors in *Arabidopsis thaliana*

AGI ID	Gene name	Substrate recognition modules	Substrates	Adaptor functions	References
BTB – BTB CRLs					
AT1G05690	BT3	Ins_element1+zf-TAZ		Gametophyte development	(124, 158)
AT1G64280	NPR1	NPR1_like_C+DUF3420+Ankyrin		Key regulator of systemic-acquired resistance	(34, 35, 50, 184, 190)
AT2G30520	RPT2	NPH3		Hypocotyl phototropism and stomatal opening	(94, 161)
AT2G41370	BOP2	Ankyrin_DUF3420	TGAs	Patterning of lateral organs	(82, 138)
AT2G46260	LRB1/POB1	BACK		Red-light signaling Defense response	(154a; M.J. Christians, D.J. Gingerich, R.D. Vierstra, unpublished)
AT3G48360	BT2	zf-TAZ		Gametophyte development	(124, 158)
AT3G51770	ETO1	TPR1+TPR2+DUF729+SNase	Type 2 ACSs	ACS turnover	(23, 208)
AT3G57130	BOP1	Ankyrin+DUF3420	TGAs	Patterning of lateral organs	(82, 138)
AT3G61600	LRB2/POB2	BACK		Red-light signaling Defense response	(154a; M.J. Christians, D.J. Gingerich, R.D. Vierstra, unpublished)
AT4G02680	EOL1	TPR1+TPR2	Type 2 ACSs	ACS turnover	(23)
AT4G31820	ENP/NPY1	NPH3		Auxin-regulated organogenesis	(59)
AT4G37610	BT5	zf-TAZ+BACK		Gametophyte development	(124, 158)
AT5G19330	ARIA	Armadillo+DUF634+KAP+ V-ATPase_H_N+HEAT	ANF2	Positive ABA response regulator	(105)
AT5G58550	EOL2	TPR1+TPR2	Type 2 ACSs	ACS turnover	(23)
AT5G63160	BT1	zf-TAZ+BACK		Gametophyte development	(124, 158)
AT5G64330	NPH3	NPH3+DUF3595		Phototropism	
AT5G67480	BT4	zf-TAZ+BACK+Sp100		Gametophyte development	(124, 158)
FBX – SCF CRLs					
AT1G12820	AFB3	LRR	AUX/IAA	Auxin receptor	(38, 142)
AT1G21410	SKP2A	LRR	E2FC/DPB	Cell division, auxin binding	(31, 98a, 99)
AT1G21760	FBP7			Protein synthesis during temperature stress	(16)
AT1G25280	ATTLP10	Tubby+DUF3527		Related to ATTLP9, ABA signaling (?)	(113)

AT1G30950	UFO	FBA3		Floral development	(46, 119, 163, 232)
AT1G43640	ATTLP5	Tubby+DUF3527		Related to ATTLP9, ABA signaling (?)	(113)
AT1G47056	VFB1	LRR+G-gamma		Plant growth and development (lateral root)	(171)
AT1G47270	ATTLP6	Tubby+DUF3527		Related to ATTLP9, ABA signaling (?)	(113)
AT1G53320	ATTLP7	Tubby+DUF3527		Related to ATTLP9, ABA signaling (?)	(113)
AT1G61940	ATTLP4	Tubby+FBD		Related to ATTLP9, ABA signaling (?)	(113)
AT1G68050	FKF1	kelch1+kelch2+PAS+PAS3+PAS4	CDF1, TOC1, PR5	Blue light receptor, circadian rhythm	(7, 137)
AT1G76900	ATTLP1	Tubby+DUF3527		Related to ATTLP9, ABA signaling (?)	(113)
AT1G77000	SKP2B	LRR	KRP1	Cell cycle	(157)
AT2G17310	SON1	FBA1+FBA3		Pathogen defense	(104)
AT2G18280	ATTLP2	Tubby+DUF3527		Related to ATTLP9, ABA signaling (?)	(113)
AT2G18915	LKP2	kelch1+kelch2+PAS+PAS3	CDF1, TOC1, PR5	Blue light receptor, circadian rhythm	(7, 170)
AT2G24540	AFR	kelch1+kelch2		Attenuated far-red-light response regulator	(79)
AT2G25490	EBF1	LRR	EIN3/EIL1	Ethylene signaling pathway	(10, 61, 74, 150)
AT2G31470	DOR	FBA1+FBA3		Negative regulator of drought resistance	(230)
AT2G39940	COI1	LRR	JAZs	JA-Ile receptor	(22, 174, 195)
AT2G42620	MAX2/ORE9	LRR		Shoot branching, strigolectone receptor (?)	(186, 214)
AT2G44900	ARABIDILLO1	Armadillo+KAP+V-ATPase_H_N+LRR		Lateral root branching	(27)
AT2G47900	ATTLP3	Tubby+DUF3527		Related to ATTLP9, ABA signaling (?)	(113)
AT3G06380	ATTLP9	Tubby+DUF3527		ABA signaling (?)	(113)
AT3G18910	ETP2	FBA1	EIN2	Ethylene signaling pathway	(152)
AT3G18980	ETP1	FBA1+Endonuc-EcoRV	EIN2	Ethylene signaling pathway	(152)
AT3G22650	CEG	FBA1+FBA3		Negatively regulates lateral root development	(41)
AT3G26810	AFB2	LRR	AUX/IAA	Auxin receptor	(38, 142)
AT3G50080	VFB2	LRR		Plant growth and development (lateral root)	(171)
AT3G54650	FBL17	LRR	KRP6/7	Germline proliferation	(77, 103)
AT3G60350	ARABIDILLO2	Armadillo+KAP+V-ATPase_H_N+YecM		Lateral root branching	(27)
AT3G61590	HAWIIAN SKIRT	kelch2		Organ fusion	(69)
AT3G62980	TIR1	LRR	AUX/IAA	Auxin receptor	(36, 38, 142, 192)

(*Continued*)

Table 2 (*Continued*)

AGI ID	Gene name	Substrate recognition modules	Substrates	Adaptor functions	References
AT4G02440	EID1			Negative regulator in phytochrome A light signaling	(39, 126)
AT4G03190	AFB1	LRR	AUX/IAA	Auxin receptor	(38, 142)
AT4G07400	VFB3	LRR		Plant growth and development (lateral root)	(171)
AT4G08980	FBW2	LRR	AGO1	ABA signaling/ARGONAUTE 1 turnover	(49)
AT4G12560	CPR30	FBA1+FBA3		Negative regulator of the defense response in *Arabidopsis*	(70)
AT4G24210	SLY1	FTH	DELLAs	GA signal perception	(40, 56, 127)
AT4G24390	AFB4	LRR	AUX/IAA	Auxin receptor	(142)
AT4G33210	SLOMO	LRR		Auxin homeostasis in the shoot meristem	(122)
AT5G18680	ATTLP11	Tubby+DUF3527		Related to ATTLP9, ABA signaling (?)	(113)
AT5G21040	FBX2	WD40+G-β		Negatively regulates phosphate starvation response	(20)
AT5G25350	EBF2	LRR	EIN3/EIL1	Ethylene signaling pathway	(10, 61, 74, 150)
AT5G48170	SNE/SLY2		DELLAs	GA signal perception	(189)
AT5G49980	AFB5	LRR	AUX/IAA	Auxin receptor	(142, 206)
AT5G57360	ZEITLUPE/ZTL	kelch1+kelch2+PAS1+PAS3	CDF1, TOC1, PR5	Blue-light receptor, circadian rhythm	(7, 182)
AT5G67250	VFB4	LRR		Plant growth and development (lateral root)	(171)
DWD – DWD CRLs					
AT1G76260	DWA2	G-β	ABI5	ABA signaling	(117)
AT2G19430	DWA1	Nup160+G-β	ABI5	ABA signaling	(117)
AT4G15900	PRL1	Nup160+G-β	AKIN10	Glucose and hormone responses	(118)
AT2G32950	COP1	RING finger+G-β		Photomorphogenesis and flowering time	(18)

Abbreviations: ABA, abscisic acid; ACS, 1-aminocyclopropane-1-carboxylic acid synthase; AUX, auxin; BTB, broad complex/tramtrack/bric-a-brac, DWD, DDB1-binding WD40 domain-containing; FBX, F-box; GA, gibberellin; IAA, indole-3-acetic acid; JAZ, jasmonic acid (JA)-ZIM; LRR, leucine-rich repeat.

recognition. In line with this possibility, the exact composition of the complete CUL4-based DWD complex is still in flux as other proteins have been implicated as potential accessory factors. The current list includes the DWD proteins CONSTITUTIVELY PHOTOMORPHOGENIC (COP)-1 and the SUPPRESSOR OF PHYA (SPA)-1 protein family working alone or together (18), DE-ETIOLATED (DET)-1 (9), and the CDD complex containing DET1 and the enzymatically inactive E2 variant COP10 in association with DDB1 (219). At present, few plant DWD proteins have been studied genetically so the range of processes affected by the corresponding DWD complexes is unclear. Connections to flowering time (18), abscisic acid signaling (117), and the turnover of the AKIN10 protein kinase (118) have been reported (**Table 2**).

Other CRLs in Plants

Besides the CUL1, -3, and -4 isoforms, yeast and animals synthesize several other CUL and CUL-like proteins that also serve as E3 scaffolds. Included in this list is the anaphase-promoting complex (APC), an 11 or more subunit ligase that contains the CUL-like protein APC2 and a RING domain-containing relative of RBX1 called APC11. Via its use of several interchangeable recognition subunits (CDC1, CDH10, and APC10), the APC is a central player in the cell cycle by removing a number of checkpoint proteins in correct sequence (144). An obvious APC complex containing APC2 and APC11 orthologs exists in plants but its organization and functions are only partially understood (57).

Phylogenetic analyses of plant genomes have detected loci encoding other CUL-like proteins in addition to the canonical types (64, 175). Although most appear by sequence alignments to express CUL truncations missing the RBX1-binding site, it remains possible that these CUL variants work alone or together with other components to scaffold novel CRL complexes. Interesting possibilities include relatives of two animal CRLs, VON-HIPPEL LINDAU (VHL) and SUPRESSOR OF CYTOKINE SIGNALING (SOCS) (14, 145). They include the CUL2 or -5 isoforms and RBX1 together with a family of VHL or SOCS box substrate adaptors, respectively (**Figure 2*c***). Both sets of adaptors are connected to the CUL2/5/RBX1 core via shared Elongin B and Elongin C heterodimeric tethers. Elongin C makes direct contact with CUL2/5 using a BTB/POZ fold similar to those found in SKP1 and BTB proteins (128, 185). Although possible relatives of CUL2/5, Elongin B, and proteins with potential VHL or SOCS-box motifs have not yet been found in any plant genome, an obvious sequence ortholog of Elongin C is universally present (M.J. Miller & R.D. Vierstra, unpublished results). *Arabidopsis* Elongin C null mutants are phenotypically normally under standard growth conditions, suggesting that if VHL or SOCS CRLs do indeed exist in plants they regulate noncritical targets.

Anaphase-promoting complex (APC): 11 or more subunit complex related to CRLs that ubiquitylates proteins important to the cell cycle

DYNAMIC REGULATION OF CRL ASSEMBLY AND ACTIVITY

CRLs must navigate through a number of competing reactions to allow smooth operation of the UPS and other Ub-dependent functions. First, the substrate adaptors must identify appropriate targets among the sea of intracellular proteins and deliver them to the correct core CUL/RBX1 ligation machinery, all in the face of stiff competition from related adaptors also engaged with substrates. Ubiquitylation must then occur rapidly via repetitive cycles of E2-Ub binding, Ub transfer, and eviction of the discharged E2 in such a way as to either mono-ubiquitylate the target or build the correct type of poly-Ub chains. And finally, when the target is sufficiently ubiquitylated, it must be released and the ligase complex disassembled to enable reuse of the CUL/RBX1 core by other awaiting adaptors. With hundreds of adaptors simultaneously delivering substrates, failure or delays of any step could inadvertently stabilize substrates or prematurely release

substrates without the requisite number of Ubs if poly-ubiquitylation was intended.

Given this complexity, it is not surprising that the abundance, assembly, and activity of the CRLs are dynamically regulated at multiple levels (14, 145). At least for some substrate adaptors, their expression can be regulated transcriptionally (2, 10, 150) and/or by microRNA-mediated (134, 142) or exonuclease-mediated (151) downregulation of the resulting mRNA. As seen for the COP1 subunit from a DWD E3 (205) and the NONEXPRESSOR OF PATHOGENESIS-RELATED GENES (NPR)-1 adaptor from a BTB E3 (184, 190), it may also be possible to manipulate CRL activity by controlling the nuclear/cytoplasm partitioning of specific components. Once a CRL is assembled, the CUL/RBX1 cores have the propensity to ubiquitylate their adaptors instead of the substrate, especially when the substrate is absent (14). This auto-ubiquitylation in turn can induce turnover of the adaptor, thus providing a broad-based mechanism to dampen the activity of individual CRLs when not needed without compromising the CUL/RBX1 core. The fact that some FBX and BTB proteins markedly increase in abundance after treating *Arabidopsis* seedlings with the 26S-proteasome inhibitor MG132 implies that adaptor regulation by auto-ubiquitylation occurs in planta (2; M.J. Christians & R.D. Vierstra, unpublished). Examples also exist where the level of a substrate adaptor is controlled by the ubiquitylation activity of another E3, thus offering a second layer of proteolytic control (14).

It appears that many CRLs can form homo- and heterodimers with dimerization stimulating Ub transfer (14). For SCF complexes, dimerization mainly occurs through a consensus D domain just upstream of the FBX sequence; the resulting dimer assumes a W-shaped suprafacial configuration in which the bound E2s potentially face both targets (e.g., 78, 173, 193) (**Figure 2*c***). For some BTB complexes, homodimerization may also occur by direct interaction between unmodified CUL3 of one monomer and the RUB1/Nedd8 moiety covalently connected to its partner (213). By either mechanism, dimerization can theoretically increase the target range of CRLs combinatorially as well as provide a mechanism to build poly-Ub chains via ubiquitylation *in trans* across the dimer. Although not yet well investigated in plants, several studies have confirmed that some BTB and DWD CRLs can be dimeric (117, 154a; M.J. Christians, D.J. Gingerich, R.D. Vierstra, unpublished). Increased target specificity may also be provided by additional factors that associate with the CRL. As an illustration, binding of the human cell cycle–inhibitor p27 to its Cdk2/Csk1 regulator helps position p27 in the binding pocket of the cognate Skp2 FBX protein for optimal ubiquitylation (17).

Clearly the most complicated and perhaps the most influential regulatory mechanism affecting CRLs involves two competing cycles driven by the CULLIN-ASSOCIATED NEDD8-DISSOCIATED (CAND)-1 protein, and RUB1/Nedd8 together with the COP9/signalosome (CSN) (**Figure 3*a***). Until recently, the roles of CSN and CAND1 were unclear owing to their paradoxical abilities to strongly inhibit CRL activities in vitro while being necessary for efficient degradation of CRL substrates in vivo (14, 87).

The first cycle revolves around RUB1 in plants and yeast or Nedd8 in animals, a Ub-fold protein most related to Ub (~60% amino acid sequence identity) with a nearly identical β-grasp structure (156). Analogous to Ub, RUB1/Nedd8 is conjugated to proteins via a three-step reaction cascade involving a heterodimeric E1, encoded by the *AUXIN-REGULATED* (*AXR*)*-1/ AXR1-LIKE* (*AXL*) gene pair and the *E1 C-TERMINALLY RELATED* (*ECR*)*-1* gene (30, 33, 37), and a single E2 encoded by paralogous *RUB1-CONJUGATING ENZYME* (*RCE*)*-1* and *RCE2* genes in *Arabidopsis* (32) (**Figure 3*a***). The E3 activity appears to be conferred by the RBX1 subunit of CRLs (72, 116), with the activated RCE1/2-RUB1 intermediate likely docking to the same surface on RBX1 that the E2-Ub intermediate bind. Given the importance of the SCF-based CRL activities in

CULLIN-ASSOCIATED NEDD-8-DISSOCIATED-1 (CAND1): protein important for dissociating and recycling CRLs following ubiquitylation of substrates

COP9/signalosome (CSN): 8-subunit complex that removes RUB1/Nedd8 bound to cullins

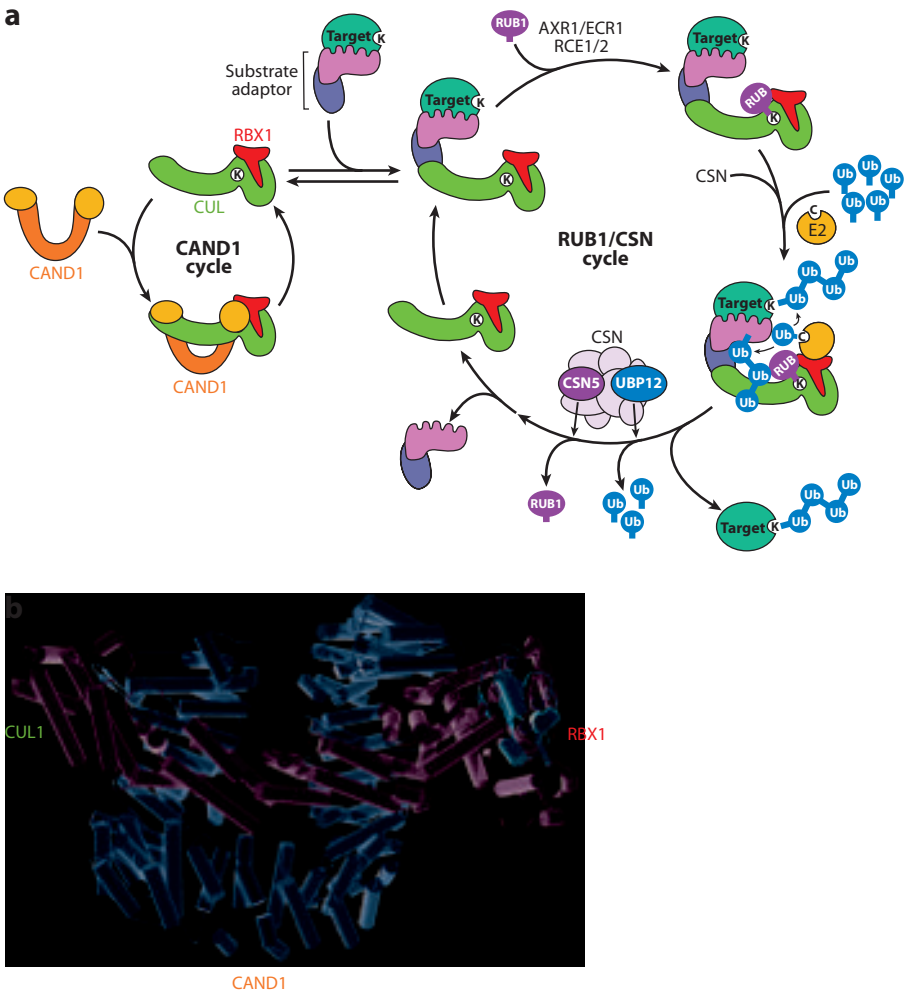

Figure 3

Proposed regulatory cycles of cullin-RING ligases (CRLs) involving CAND1 and RUB1-CSN-mediated RUBylation/de-RUBylation of the cullin (CUL) subunit. (*a*) Diagrams of the regulatory cycles. Via a transient and reversible binding of CAND1 to the CUL/RBX1 catalytic core, a dynamic pool of uncommitted CRLs is maintained in the cell. Occlusion of both the adaptor-binding and RUB1-binding sites by the U-shaped CAND1 prevents CRL assembly. Various adaptors then identify cellular substrates and recruit them to unsequestered CUL/RBX1 cores to generate an active CRL complex that enters the RUB1/CSN cycle. Through the action of the AXR1/ECR1 E1 heterodimer, the RCE1/2 E2, and an E3 activity provided in part by RBX1, RUB1 is attached to a positionally conserved lysine (K) at the C-terminal end of the CUL, which in turn helps activate the CUL/RBX1 ubiquitin (Ub) ligase activity. Ubs are subsequently added to the substrate and sometimes to the substrate adaptor (auto-ubiquitylation), especially if no substrate is present. The ubiquitylated substrate is released and often degraded by the 26S proteasome. Either before or after the substrate is fully ubiquitylated, the engaged CRL complex associates with the eight-subunit COP9/signalosome (CSN). Through the action of the de-RUBylating activity provided by the CSN5 subunit and the deubiquitylating enzyme (DUB) activities provided in part by the associated DUB UBP12, RUB1 and Ubs bound to the CUL and the adaptor, respectively, are released from the CRL. Final dissociation of the substrate adaptor then allows the CUL/RBX1 core to re-enter the CAND1 and RUB1/CSN cycles for eventual reuse. (*b*) Three-dimensional ribbon model of CAND1 in a ternary complex with human CUL1 and RBX1 (adapted from Reference 67).

auxin signaling, all *Arabidopsis* mutants compromising RUB1/Nedd8 conjugation have altered auxin sensitivity (87). A second factor, DEFECTIVE IN CULLIN NEDDYLATION (DCN)-1, also drives RUB1/Nedd8 conjugation in yeast and animals (112). A likely DCN1 ortholog was recently identified in *Arabidopsis* by a genetic screen for resistance to an auxin antagonist (11), but its role in plant RUBylation remains to be confirmed.

Although a few other RUBylation substrates have been proposed (14, 87), the main targets are the CULs, with the three canonical *Arabidopsis* CULs (CUL1, -3, and -4) confirmed to be modified in vivo (13, 19, 32, 54, 118, 210). RUB1 is covalently attached to a positionally conserved lysine within a near invariant VRI/VMK motif, which is adjacent to the RBX1-binding site in the CUL three-dimensional structure (32, 210). Recent biochemical studies with nonplant components showed that this attachment improves the affinity of the CUL/RBX1 cores for the activated E2-Ub intermediate and induces a conformational change in the CUL, which tilts the E2 closer to the substrate-binding pocket (45, 160, 162).

RUB1/Nedd8 addition to CULs is reversed by the CSN (**Figure 3*a***). This previously enigmatic eight-subunit complex was first identified from a screen for light-perception mutants in *Arabidopsis* (211). Whereas null mutants affecting each of the CSN subunits are embryo lethal, weaker *csn* alleles display a strong constitutive photomorphogenic phenotype even when grown in dark and have altered auxin sensitivity. Subsequent studies revealed that the CSN is evolutionarily conserved and distantly related to the regulatory particle of the 26S proteasome (55, 66). De-RUBylation of CULs is directed by the CSN5 subunit, a zinc-dependent metalloprotease containing a JAB1/MPN/Mov34 (JAMM) catalytic site (28, 123). CSN5 is encoded by two essential genes in *Arabidopsis*. Single *csn5a* or *csn5b* mutants or weak *csn5a csn5b* double mutants induce pleiotropic defects similar to those defective in RUB1 conjugation (75, 76, 172). One or more DUBs (UPB12 in humans) associate substoichiometrically with the CSN; one of their likely functions is to stabilize CRLs by removing Ubs that become attached autocatalytically to the adaptors (168, 234).

The second cycle of CRL regulation is driven by CAND1, a 120-kDa protein containing a long string of HEAT-repeat helical rods (67). The *Arabidopsis* CAND1 ortholog was independently identified by several groups, with subsequent genetic studies showing that it, like RUB1 and CSN, is important for one or more Ub-mediated steps affecting auxin signaling and photomorphogenesis (1, 21, 25, 53). Where tested, CAND1 has the capacity to interact with all canonical CUL types (e.g., 129). In vitro, this binding readily inhibits CRLs by dissociating the adaptor module from the CUL1/RBX1 core. The three-dimensional structure of human CAND1 bound to CUL1/RBX1 (67) revealed that it works as a clamp with its remarkable U-shaped structure wrapping around the elongated CUL1 surface (**Figure 3*b***). A β hairpin at its C terminus partially occludes the adaptor-binding site in CUL1, thus inhibiting the interaction of CUL1 with the SKP1/FBX subcomplex. The N-terminal end of CAND1 packs against the surface of CUL1 to cover the RUB1 conjugation site, effectively blocking this modification as well. Via these interactions, CAND1 simultaneously interferes with CUL RUBylation and docking of the substrate adaptor to the CUL/RBX1 core. Conversely, RUB1/Nedd8 addition interferes with CAND1 binding to the core.

Numerous hypotheses have attempted to explain the paradoxical functions of RUBylation/de-RUBylation and CAND1 binding on CRL action (see 14, 87). The most plausible model by Schmidt et al. (168) posits that their interplay is essential to maintain a dynamic population of free CUL/RBX1 cores and for subsequent adaptor/substrate loading and ubiquitylation. As shown in **Figure 3*a***, CAND1 binding to non-RUBylated CUL/RBX1 catalytic cores is viewed as transient and reversible and is

designed to maintain a dynamic population of inert cores without substrate adaptors. Once a substrate adaptor along with its substrate becomes engaged with a free CUL/RBX1 core, the CUL is RUBylated, which prevents further interference by CAND1. Stimulated by the modification, the RUB1/Nedd8-modified holo-CRL then binds to the CSN, using it as a platform for safe and efficient ubiquitylation of the substrate. If the adaptor becomes auto-ubiquitylated inadvertently, this addition is reversed by one or more DUBs bound to the CSN. Given the energy present in the unstable E2-Ub intermediate and the potential wobble of the bound substrate, such spurious Ub transfer may be unavoidable. Once the substrate is appropriately modified, and presumably released, the CRL is dissociated by CSN-induced de-RUBylation; the de-RUBylated CUL/RBX1 core then re-enters the CAND1-binding/release cycle. Through these opposing cycles, CRLs are maintained in a dynamic equilibrium, which allows many competing adaptors to deliver substrates to the ubiquitylation machinery while encouraging sufficient engagement with the substrate as poly-ubiquitylation proceeds but discouraging and/or reversing auto-ubiquitylation.

The CAND1-RUB1/CSN cycles would be particularly advantageous to plants and animals given the plethora of possible substrate adaptors for each CRL type (60, 65, 91, 118). Accordingly, higher-order eukaryotes deficient in the CAND1 and RUB1/Nedd8 cycles are more broadly affected than comparable yeast mutants (14, 87). Although support for such a model in plants is still forthcoming, several genetic studies in *Arabidopsis* are consistent with its framework. These include data showing that (*a*) only a small fraction of unmodified CUL1 associates at steady state with CAND1 (53), (*b*) mutants in CAND1 that abrogate CUL1 binding inversely affect the assembly of SCF complexes (229), and (*c*) mutants that alter the RUB1/Nedd8 cycle and the CSN both negatively affect SCF E3 activities (25, 72, 75).

Even with this rudimentary understanding, key parts of the CAND1/RUB1-CSN cycles are still not clear. For instance, are all adaptors regulated by the two cycles? It has been proposed that only a subset of FBX proteins, which use a positionally conserved proline in their FBX domains to directly bind CUL1, are stabilized by the CSN (168). This critical proline is absent in ~30% of the plant FBX proteins, including some known to be functionally important, suggesting that they are regulated differently. Do adaptors engage substrates before or after docking with the CUL1/RBX1 core? Are there additional factors (chaperones?) that prevent CRL E3s from becoming overwhelmed with free adaptors? What signal(s) trigger RUBylation and de-RUBylation of the CUL? Are there other activities associated with the CSN besides de-RUBylation? How does the complex know when a sufficient number of Ubs with the right topology is added to the substrate? And finally, by what mechanism is the CRL disassembled after ubiquitylation of the substrate? Hopefully, the recent development of in vitro CRL ligation systems will soon help address these questions (e.g., 146, 148, 160, 207).

DIVERSE FUNCTIONS OF CRLS

Given the extraordinary number of CRL substrate adaptors in plants, it is not surprising that the resulting E3s have now been connected to nearly all facets of plant physiology and development. Their versatility is exemplified by their ability not only to detect various protein targets, but also to bind small molecule hormones, chromophores, and sugars with high specificity. Most of the plant CRL adaptors do not have obvious relatives in yeast and animals, implying that many have evolved with plant-specific targets and functions. The current list of characterized CRL adaptors with ascribed functions, mainly deduced by forward- and reverse-genetic strategies in *Arabidopsis*, is provided in **Table 2**. Owing to space limitations, only a few of these are described below to highlight the breadth of events under CRL control.

TRANSPORT INHIBITOR RESPONSE-1 (TIR1): FBX protein that is the signal receptor for auxin

CORONATINE-INSENSITIVE-1 (COI1): FBX protein that is the signal receptor for jasmonic acid

CRLs Participating in Hormone Perception

The application of various genetic strategies, especially using *Arabidopsis*, has recently uncovered how a handful of the plant hormones work at the molecular level with the remarkable discovery that several exploit the UPS in general and CRLs specifically in signal transmission (164, 203). The most surprising were the findings that several FBX proteins work as either hormone receptors or coreceptors. The first example came from an understanding of how the hormone auxin or indole-3-acetic acid (IAA) is perceived. Auxin is mainly synthesized in meristems and transported via a sophisticated transport system to generate local maxima that direct cell development and elongation in response to numerous internal and external cues (201). The central regulators are a family of auxin response factors (ARFs), which either promote or inhibit the transcription of a variety of auxin effector genes (164). In the absence of auxin, ARFs are repressed by direct association with an equally large family of AUXIN (AUX)/IAA repressors together with their TOPLESS corepressor. Auxin then stimulates the proteolytic removal of AUX/IAA proteins by the UPS thus allowing ARFs to act.

Genetic studies implicated the conserved Domain II sequence in AUX/IAA proteins and the LRR-containing FBX protein TRANSPORT INHIBITOR RESPONSE (TIR)-1 in auxin-mediated AUX/IAA turnover (73, 228), but how was a mystery. After exploring various possibilities, researchers found that TIR1 directly binds Domain II and that this binding requires auxin, thus implicating the SCF^{TIR1} complex as the previously enigmatic auxin receptor (36, 101). The crystal structure of the quaternary complex of TIR1, SKP1(ASK1), auxin, and the Domain II peptide from IAA7 confirmed this proposal (192). The structure revealed a solenoid-shaped pocket formed by the 18-tandem LRRs in TIR1 cradling the Domain II degron with the auxin beneath acting as a "molecular glue" to strengthen the interaction (**Figure 4*a***). Synthetic auxins with distinct ring structures can also fit into this somewhat promiscuous hydrophobic pocket, thus explaining in molecular terms why they also have auxin activity in planta (80, 192). Surprisingly, the bottom of the TIR1 pocket contains a tightly bound inositol hexaphosphate ($InsP_6$ or phytic acid) that helps maintain the solenoid LRR configuration via a set of electrostatic interactions with neighboring lysines/arginines. Phylogenetic studies identified five other FBX proteins related to TIR1 in *Arabidopsis* [AUXIN-BINDING FBX (AFB)-1–5]; subsequent reverse-genetic and auxin-binding studies demonstrated that SCF^{AFB1-5} also work combinatorially in auxin perception (38, 142, 206).

The detection of LRR-containing FBX proteins similar to TIR1/AFB1–5 in *Arabidopsis* suggested that other SCF E3s could also be small molecular sensors (60, 142). One likely candidate was CORONATINE-INSENSITIVE (COI)-1, an FBX protein initially discovered from a genetic screen for resistance to coronatine (216), a potent phytotoxin from *Pseudomonas syringae* that is structurally related to the oxilipin hormone jasmonic acid (JA). A glimpse of COI1 function was first provided by the identification of its targets, a family of JA-ZIM (JAZ) transcriptional regulators that repress JA responses by binding to and inhibiting the MYC2 transcription factor (22, 195). Presumably, after ubiquitylation-mediated degradation of JAZ proteins by SCF^{COI1}, MYC2 is free to direct JA-dependent transcription. Like auxin and TIR1, the binding of the bioactive form of JA, JA-isoleucine (Ile), to COI1 greatly potentiates the association of JAZ proteins to COI1 in vitro, strongly suggesting that SCF^{COI1} is a JA receptor (100). In fact, the COI1/JAZ complex is a 1000-fold tighter if coronatine is used instead of JA-Ile, in agreement with its greater potency in planta (100). The interaction between COI1 and JAZ proteins was mapped to a C-terminal JAZ degron that is essential for COI1-mediated turnover.

Proof that SCF^{COI1} is the actual JA receptor was recently confirmed from the x-crystallographic structure of the quaternary

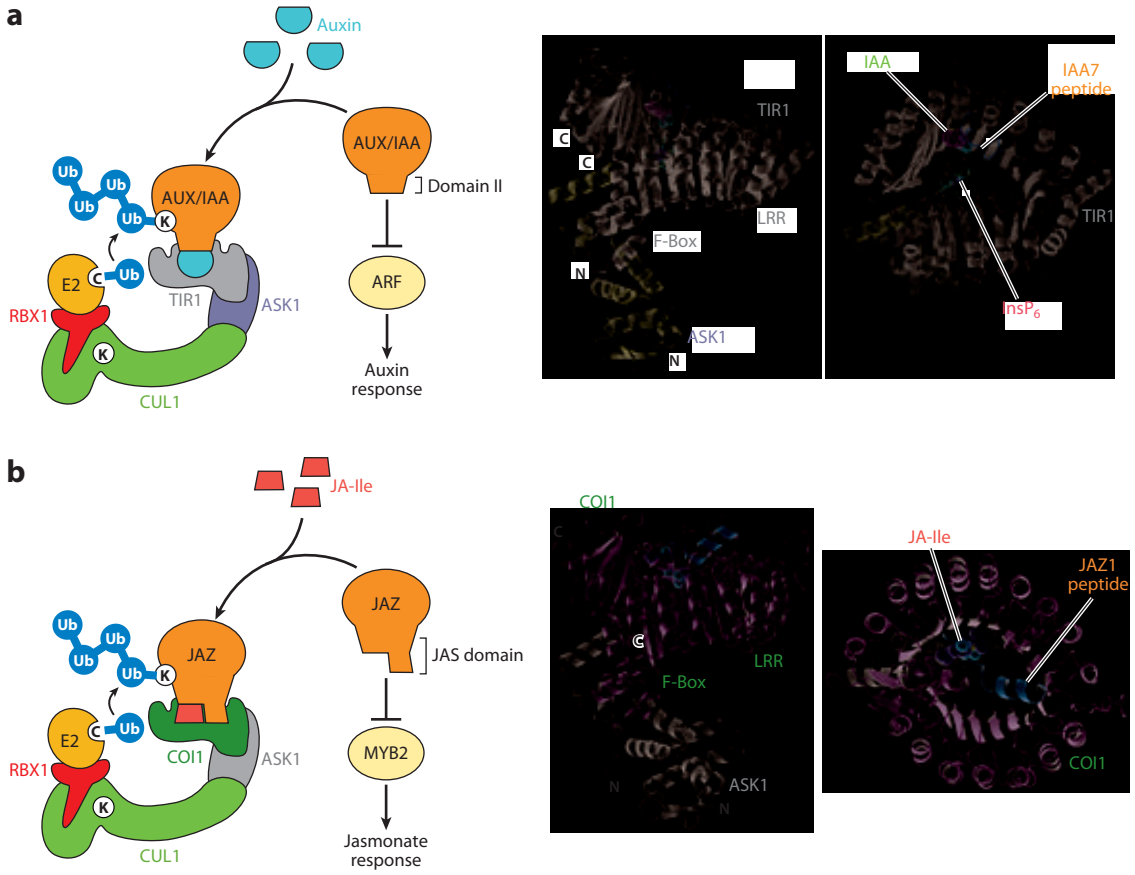

Figure 4

Structure of the (*a*) SCF^{TIR1} and (*b*) SCF^{COI1} hormone receptors. Left diagrams show the organizations and mechanism of action for the SCF^{TIR1} and SCF^{COI1} E3 complexes following indole-3-acetic acid (IAA) and jasmonic acid (JA)-Ile binding, respectively. Pictures on the right show side and top views the corresponding three-dimensional models of the TIR1 and COI1 proteins assembled with hormone, target protein degron, and SKP1(ASK1). The TIR1 structure contains IAA, the degron peptide from IAA7, and $InsP_6$. The COI1 structure contains JA-Ile and the JAZ1 peptide. The bottom of the COI1 binding pocket contains $InsP_5$, which is not shown. N, N terminus; C, C terminus (adapted from References 192 and 174).

complex consisting of COI1, SKP1(ASK1), JA-Ile, and the JAZ degron from JAZ1 (174). Analogous to TIR1, the 18-tandem LRRs of COI1 create a solenoid-shaped pocket that binds JA-Ile in a central cavity (**Figure 4*b***). However, unlike TIR1, COI1 employs inositol pentakisphosphate ($InsP_5$), not $InsP_6$, in the cavity's base to stabilize the looped LRR configuration. Recognition of the JAZ1 target by COI1 also slightly differs from the molecular glue mechanism used by TIR1 to recognize AUX/IAA proteins. Instead of forming a singular FBX/hormone/degron sandwich, the helical section of the JAZ degron, which is highly conserved among JAZ proteins, binds to the COI1 pocket while an adjacent highly degenerate and flexible section binds to JA-Ile, thus creating a bipartite recognition interface (174).

Do other CRL adaptors act as receptors for plant hormones? One intriguing case is MORE AXILLARY GROWTH (MAX)-2/ORESARA (ORE)-9, an LRR-containing FBX protein similar to TIR1 and COI1. MAX2/ORE9

was originally identified in mutant screens for proteins defective in shoot branching (186) and senescence (214), with subsequent studies also revealing a role in seed germination. Although the target(s) of $SCF^{MAX2/ORE9}$ is still unclear, this E3 appears to participate in a signaling pathway downstream of the newly discovered plant hormone strigolactone (68, 198). Because *max2/ore9* mutants poorly respond to strigolactone, an appealing possibility is that $SCF^{MAX2/ORE9}$ stimulates the turnover of a negative regulator of strigolactone perception after binding the hormone.

Although not acting as the direct receptor, one or more CRLs participate in perception of the gibberellin (GA) family of steroid hormones that promote cell division and seed germination. GA signaling requires removal of a family of DELLA transcriptional repressors, which are targeted by SCF E3s assembled with the FBX proteins SLEEPY (SLY)-1/SNEEZY (SNE)-1 in *Arabidopsis* (5a, 40, 56, 127, 189) and GA-INSENSITIVE DWARF (GID)-2 in rice (166). Unlike SCF^{TIR1} and SCF^{COI1}, the $SCF^{SLY1/SNE1/GID2}$ complexes do not directly bind GAs. The hormone is first recognized by the nuclear-localized GA receptor GIBBERELLIN INSENSTIVE DWARF1 (GID1). The GA-GID1 complex then binds to DELLA proteins, which in turn enhances the recognition of DELLAs by $SCF^{SLY1/SNE1/GID2}$ probably by altering the conformation of the C-terminal GRAS domain of the DELLA proteins (133, 176). In this way, GA serves as a coregulator of DELLA turnover.

Hormone Synthesis and Signaling

In addition to direct perception of hormones, CRLs can be intimately involved in the regulation of hormone synthesis and their downstream signaling cascades (**Table 2**). Nowhere is this more evident than in the synthesis and response of *Arabidopsis* to the gaseous hormone ethylene, which is controlled by several CRL E3s working at multiple levels. Synthesis of the hormone is rate limited by a family of 1-aminocyclopropane-1-carboxylic acid (ACC) synthases (ACSs) that convert S-adenosylmethionine to the immediate ethylene precursor ACC (15, 203). Type-1 and type-2 ACSs are constitutively degraded in a UPS-dependent manner in the absence of ethylene, with ethylene abrogating this turnover to stimulate ethylene production by positive feedback. Degradation is regulated by distinct E3s recognizing unique C-terminal motifs in each ACS type. For type-1 ACSs, ethylene-induced phosphorylation of this motif likely blocks E3 recognition (98, 224). For type-2 ACSs, breakdown is directed by a family of three *Arabidopsis* BTB E3s that are assembled with the adaptors ETHYLENE OVERPRODUCER (ETO)-1, ETO1-like (EOL)-1, and EOL2 (23, 208). BTB^{ETO1} plays the dominant role in young seedlings, with the actions of BTB^{EOL1} and BTB^{EOL2} becoming phenotypically obvious in *eto1 eol1 eol2* triple-mutant backgrounds.

Perception and response to ethylene are also affected by the UPS, with at least two different SCF E3s playing major roles in *Arabidopsis* (2, 10). The dominant regulatory step involves turnover of the transcription factors ETHYLENE-INSENSITIVE (EIN)-3 and EIN3-like (EIL)-1 by SCF complexes assembled with EIN3-binding FBX protein (EBF)-1 or EBF2 (61, 74, 150). Orthologs of EBF1/2 are widely distributed in the plant kingdom, indicating that the corresponding SCF E3s represent a central feature in ethylene signaling (91). In the absence of ethylene, EIN3/EIL1 is rapidly degraded following $SCF^{EBF1/2}$-mediated ubiquitylation to keep the levels of these transcription factors low. Ethylene interfers with the recognition of EIN3/EIL1 by $SCF^{EBF1/2}$ followed by possible auto-ubiquitylation and subsequent removal of EBF1/2 (2, 10). Ethylene-induced increases in EIN3/EIL1 then enhance transcription of a variety of ethylene response genes. The block in EIN3/EIL1 recognition was proposed to be mediated by EIN3/EIL1 phosphorylation via a mitogen-activated-protein kinase pathway (223), but more recent studies have questioned this effect (2).

Within the ethylene response, EBF1 and EBF2 appear to have distinct roles. Whereas EBF1 is constitutively expressed and responsible for maintaining low levels of EIN3/EIL1 in the absence of hormone, EBF2 expression is upregulated by an EIN3-mediated mechanism that then allows it to play a more prominent role in removing excess EIN3/EIL1 during the latter stages of the ethylene response and after the hormone dissipates (10, 110, 150). Levels of *EBF1* and *EBF2* mRNA are also negatively controlled posttranscriptionally by the exoribonuclease EIN5/XRN4 to further modulate EIN3/EIL1 protein accumulation (139, 151).

A second, less well-understood step in *Arabidopsis* ethylene signaling involves the intermediate signaling factor EIN2, an endoplasmic reticulum (ER)-bound protein with similarity to NRAMP metal transporters. Like EIN3/EIL1, EIN2 is degraded in the absence of ethylene but dramatically stabilized by the hormone. Breakdown is directed by a pair of SCF E3s assembled with the FBX proteins EIN2-targeting protein (ETP)-1 or ETP2 (152). Surprisingly, whereas the C-terminal domain in EIN2 that binds ETP1/2 is conserved throughout the plant kingdom, obvious ETP1 and ETP2 orthologs can be found only in the closely related *Arabidopsis lyrata* species (91, 209). The lack of conservation for ETP1/2 suggests either that this regulatory step recently evolved and is not widespread in the plants, or that other E3s assume this role in less-related species.

CRLs and Light Perception

Besides serving as chemical receptors, a unique class of CRLs that function as photoreceptors exists in plants and animals. In *Arabidopsis*, these E3s are assembled with the FBX proteins ZEITLUPE (ZTL), flavin-binding kelch repeat (FKF)-1, and LOV-kelch protein (LKP)-2 that bear at their N termini an evolutionary conserved light-oxygen-voltage (LOV) domain that binds flavin mononucleotide (FMN) (137, 170, 182). Light detection is achieved by photo-induced formation of an FMN-cysteine adduct. The adduct conformationally alters the LOV domain, with the change presumably propagated to the β-propeller recognition module formed by the kelch domain repeats. Collectively, ZTL, FKF1, and LKP2 help entrain various plant circadian rhythms to blue light by controlling the stability of three core clock components, TIMING OF CAB EXPRESSION (TOC1)-1, pseudoresponse regulator (PRR)-5, and CYCLING DOF FACTOR (CDF1)-1, in a circadian-dependent manner.

The levels of ZTL and FKF1 fluctuate during the day/night cycles by binding to an accessory component GIGANTEA (GI), whose expression is circadian rhythmic (106, 167). GI binding protects ZTL from UPS-mediated degradation with this binding enhanced by photoactivation of ZTL, thus generating a robust circadian oscillation of ZTL levels against a background of constitutive *ZTL* gene expression. FKF1 similarly oscillates by GI binding and photoactivation, which is accentuated further by circadian regulation of *FKF1* gene transcription. SCF^{ZTL} ubiquitylates TOC1 and PRR5, thus cycling the abundance of these related clock components in an inverse pattern relative to the E3 (102, 106). Conversely, SCF^{FKF1} and possibly SCF^{LKP2} ubiquitylates CDF1, a repressor of CONSTANS (CO) expression (93, 167). Removal of CDF1 allows CO levels to rise to help elicit a number of photoperiodic responses under CO control (e.g., flowering time). In agreement with the use of similar substrate recruitment kelch repeats, FKF1 and LKP2 may also recognize TOC1 and PRR5 (7). The dual actions of SCF^{ZTL} and $SCF^{FKF1/LKP2}$ produce robust oscillating patterns of clock factors and outputs that are easily entrained to the day/night cycles by chromophore photoactivation.

Other CRLs do not act directly in photoreception but in the downstream steps directing photomorphogenesis (**Table 2**). The BTB E3s formed with the NPH3 and LRB1/2 BTB adaptors appear to play important roles in regulating blue- and red-light perception by controlling signaling from phototropin-1 (143) and the abundance of phytochrome-B

photoreceptors (M.J. Christians, D.J. Gingerich, R.D. Vierstra, unpublished), respectively. Further downstream are SCF E3s assembled with the ATTENUATED FAR-RED RESPONSE (AFR) and EMPFINDLICHER IM DUNKELROTEN LICHT (EID)-1 FBX proteins that inversely modulate PhyA signaling (39, 79). COP1 plays a central role in the turnover of numerous photomorphogenic regulators, including CO and LONG HYPOCOTYL (HY)-5 (97, 141). Although originally classified as a RING-type E3, more recent studies suggest that COP1 associates with DWD complexes (18, 19).

Control of the Cell Cycle and Development

The UPS is a central effector of the plant cell cycle where it helps sequentially remove numerous checkpoint proteins such as cyclins, cycle-dependent kinase (CDK) inhibitors, and securins as the cell cycle proceeds (17, 144). Accordingly, a number of *Arabidopsis* E3s have been implicated, including HECT, APC, and CRL ligases (43, 57, 77, 103, 159). The contributions of SCF E3 were first inferred from the phenotypes of *Arabidopsis skp1*(*ask1*) mutants, which display strong defects in male gametophyte meiosis (220).

The G1/S phase transition in *Arabidopsis* is tightly controlled by the activity of CDKA:1, whose action must be derepressed by removal of its CDK inhibitors, KIP-related protein (KRP)-6 and KRP7. Recent genetic studies showed that an SCF complex assembled with the FBX adaptor FBL17 specifically directs KRP6/7 turnover in the male germline (77, 103). FBL17 is nuclear localized and significantly upregulated in the pollen generative cell just prior to the second mitotic division that forms the two male gametes. Loss-of-function *fbl17* mutants prevent this division by failing to remove KRP6/7. The resulting single male gamete can fertilize only the egg cell, with the resulting embryo aborting early in embryogenesis owing to a lack of endosperm (77, 103). The FBX protein S-phase kinase-associated protein 2A (SKP2A) also appears to regulate cell division through ubiquitylation of the cell cycle transcription factors E2FC and DPB (98). Intriguing, recent data indicate that SKP2 can also bind auxins directly, thus potentially identifying another CRL-based receptor for this hormone (98a). One or more DWD-type CRLs have also been connected to plant cell division from the discovery that RNA-interference mutants of *CUL4* undergo altered rounds of endoreduplication in *Arabidopsis* trichomes (159).

A number of forward- and reverse-genetics studies have linked specific CRLs to various developmental processes. In fact, one of the first *Arabidopsis* FBX proteins discovered was UNUSUAL FLORAL ORGANS (UFO), whose deletion severely affects floral homeosis (163). The types of phenotypic defects observed, ranging from cell/tissue transformations to ectopic organs, suggest that key components controlling meristem integrity, cell specification, and cell differentiation are modulated by these CRLs (**Table 2**). At present, the identities of the developmental regulators ubiquitylated by these CRLs are largely unknown.

Role of CRLs in Protein Quality Control

Whereas most CRLs target correctly folded, functional proteins, it is also apparent that a subset of CRLs has housekeeping roles by removing misfolded counterparts. This action is particularly important for the endomembrane trafficking system, where aberrant polypeptides are retained in the ER instead of reaching the final destination(s) of their functional counterparts. To facilitate their removal, these aberrant polypeptides are transported back into the cytosol via retrograde mechanisms where they are removed by an ER-associated degradation (ERAD) quality-control pathway that involves both Ub and the 26S proteasome (155). In mammals, two FBX proteins, Fbs1 and Fbs2, play key roles in ERAD-mediated turnover by detecting abnormally folded glycoproteins with high mannose-type N-glucans (225, 226). A small collection of FBX proteins with

similar lectin-like recruitment modules are present in *Arabidopsis* and other plants (60, 96, 114), suggesting that an analogous SCF-dependent ERAD pathway works in the plant kingdom.

Self-Recognition During Reproduction

To avoid inbreeding, many flowering plants have developed incompatibility barriers that prohibit self-pollination, at least two of which exploit the UPS (89, 154). One major self-incompatibility (SI) mechanism, which is used by *Solanaceae*, *Plantaginaceae*, and *Rosaceae* species, involves a polyallelic ribonuclease (*S-RNase*) gene within the *S* locus (88, 231). The encoded polymorphic RNases are expressed in the pistil of the flower and accumulate in the transmitting tract where they enter the growing pollen tubes of both self and nonself pollen. The imported S-RNase is cytotoxic to self pollen tubes by degrading their RNAs, thus inducing SI by blocking sperm delivery. Remarkably, nonself pollen tubes escape this growth arrest by selectively degrading the S-RNase, using a collection of SCF complexes assembled with a presumably equally polymorphic family of *S*-locus FBX proteins (SLF for *Solanaceae* and *Plantaginaceae* and SFB for *Rosaceae*) (90, 153, 177). The haplotype-specific alleles of both the *S-RNase* and *SLF/SFB* genes are tightly linked in the *S* locus to maintain the SI barrier from generation to generation.

The SLF adaptors from *Antirrhinum hispanicum* (*Plantaginaceae*) appear to be tethered to CUL1 via a novel SKP1 bridge (SSK1); both SLFs and SSK1 are specifically expressed in pollen tubes (92, 154). In *Petunia inflata* (*Solanaceae*), an alternative SCF^{SLF} complex that incorporates a novel S-RNase-binding protein (SBP)-1 was proposed (89, 178). Analysis of three SI species from the *Petunia* genus revealed that the *S*-locus can contain multiple types of polymorphic *SLF* genes, each of which encodes an SLF protein designed to target a subset of S-RNase alleleic variants (111a). This collection presumably collaborates to recognize and remove the entire suite of non-self S-RNases during a compatible pollination (111a). Biochemical analysis of the petunia SI system showed that its haplotype-specific SLFs preferentially interact with nonself S-RNases both in vivo and in vitro (89, 90, 111a). UPS-mediated S-RNase breakdown has also been demonstrated in vitro, but no S-RNase haplotype specificity was observed (89, 89a). This lack of specificity could reflect an inherent non-specificity of the in vitro system, the absence of a key specificity component, or the possibility that the turnover reflects basal S-RNase proteolysis mediated by SBP1 (89a). Reduced amounts of S-RNases in compatible pollen tubes from *Solanum chacoense* (*Solanaceae*) imply that this SCF^{SLF}-mediated degradation also occurs in vivo (120).

It remains unclear how self SCF^{SLF} E3s are able to recognize nonself S-RNases but avoid targeting those from self. One model proposes that a competitive interaction among domains in the S-RNases and the SLF adaptors sterically interferes with binding and ubiquitylation of self S-RNases (88). Other less complicated possibilities are that the S-RNase and SLF from self cannot interact, whereas self/nonself pairs can (111a), or that the repertoire of SCF^{SLF} E3s can recognize both self and nonself S-RNases but the self S-RNase preferentially triggers FBX auto-ubiquitylation and subsequent turnover, thus allowing the self isoform to survive. Given the subtle differences among allelic S-RNases and their SLF/SFB partners, understanding the recognition mechanism involved should illuminate the sophisticated methods used by CRLs to identify appropriate targets with high specificity.

Interplay Between Pathogens and Plant Hosts

Plants are in a continuous battle with numerous viral, bacterial, and fungal pathogens that seek suitable hosts for food and shelter. It is becoming increasingly obvious that the UPS in general and CRLs in particular provide a major defense barrier for plants as well as offer countermeasures for the intruder. One

key host defense mechanism involves the BTB E3 assembled with the NPR1 adaptor (47). NPR1 helps mediate systemic-acquired resistance to pathogens via the defense hormone SA, presumably by ubiquitylating a currently unknown substrate.

The BTB^{NPR1} complex is regulated at multiple levels for which changes in the cellular redox status are a key signal. In the absence of pathogens, most NPR1 becomes sequestered in the cytoplasm as free oliogmers created by S-nitrosylation-facilitated intermolecular disulfide bridges (190). Upon pathogen challenge, SA levels rise, which in turn triggers monomerization of NPR1 via thioredoxin-mediated reduction of the bridges. The NPR1 monomers then enter the nucleus where they assemble with CUL3/RBX1. The resulting BTB^{NPR1} complex presumably degrades a negative regulator of SA action. Monomeric NPR1 is cleared from the nucleus by a UPS-dependent process (possibly auto-ubiquitylation) that requires phosphorylation of NPR1 (184). This regulated turnover/localization could prevent untimely activation of SA signaling by keeping active NPR1 levels low in the absence of SA and then maintain a reasonable level of active NPR1 as SA levels rise. Other host CRLs involved in pathogen defense include the tobacco Avr9/Cf-9-INDUCED F-BOX (ACIF)-1 protein that induces a general hypersensitive defense response to a range of viral and bacterial pathogens (199), and the *Arabidopsis* FBX protein SUPPRESSOR OF NIM1 SIGNALING (SON)-1, which confers resistance by an SA-independent mechanism (104).

Numerous plant pathogens have also co-opted the UPS for their own benefit. The best example is the bacterial pathogen *Agrobacterium tumefaciens*, which exploits an FBX protein encoded by its *VIRULENCE F* (*VirF*) locus to help promote infection (197). Upon plant contact, VirF is injected into the host cell along with the single-stranded T-DNA via a type-three secretion system (T3SS). Once inside, VirF enters the nucleus and assembles with plant components to form an SCF^{VirF} complex. The resulting E3 then triggers breakdown of the *A. tumefaciens* VirE2 protein and the host VIRE2-INTERACTING PROTEIN 1 (VIP1) protein, both of which coat and help chaperone the T-strand DNA into the nucleus but must be removed before chromosomal integration of the T-strand DNA (197). Surprisingly, *A. tumefaciens* strains lacking VirF can induce expression of a host alternative that can functionally replace VirF in mediating VirE2 and VIP1 breakdown (227).

Other pathogen-encoded CRL components include the P0 FBX protein from pathogenic poleroviruses, the FRP1 FBX protein from *Fusarium oxysporum* (48), and the GALA FBX proteins from the bacterial pathogen *Ralstonia solanacearum*. Upon infection, P0 assembles with host CUL1, RBX1, and SKP1 to form an SCF complex that directs the degradation of ARGONAUTE (AGO)-1, a key component of the plant RNA-induced silencing complex (8, 12). Suppression of AGO1 by SCF^{P0} subsequently protects viral genome replication from host-mediated viral-silencing mechanisms. Coincidently, *Arabidopsis* AGO1 levels are also regulated by an endogenous FBX FBW2 (49). The seven-member GALA family belongs to the collection of T3SS proteins from *R. solanacearum* that enters the plant host upon infection (4). Once inside, they presumably assemble into SCF^{GALA} complexes directed to degrade one or more plant defense proteins using their LRRs for substrate identification. This proteolysis appears critical as removal of the FBX domain in GALA7 severely dampened infection of *Medicago truncatula* by the bacterium (5).

In addition to injecting CRL adaptors into plants, plant pathogens may also exploit CRL regulators. As mentioned above, a good example is the JA mimic coronatine, which is synthesized by *P. syringae* to overstimulate the JA receptors SCF^{COI1} (100). Another novel strategy comes from geminiviruses which use their C2 protein to dampen CRL-mediated defense responses. The C2 protein appears to work by binding to and attenuating the de-RUBylation activity of the CSN5 subunit of the CSN

complex thereby interfering with many CRLs simultaneously by blocking their RUBylation cycle (R. Lozano-Durán & E.R. Bejarano, unpublished). A third strategy may involve relatives of the global regulator CYCLE INHIBITING FACTOR (Cif), a T3SS protein synthesized by the animal pathogens *Burkholderia pseudomallei* and enteropathogenic *Escherichia coli* (29). Cif is a deamidase that specifically and efficiently deamidates Gln-40 in both Ub and RUB1/Nedd8 upon entry into the host. This modification effectively abrogates the activity of all host CRLs that require RUB1/Nedd8 attachment and suppresses much of the rest of the UPS by impairing poly-Ub chain formation.

EVOLUTION OF CRL SUBSTRATE ADAPTORS

Well before genetic analyses confirmed the prominence of CRLs in plant biology, their significance was presaged by various phylogenetic studies on the respective substrate adaptors, which revealed that many hundreds to a thousand CRLs may be expressed in some plant species (60, 64, 65, 91, 96, 118, 217). Whereas the numbers of possible plant BTB and DWD CRLs are comparable with those in most metazoans, the number of possible plant SCF CRLs is typically much larger (**Table 1**). For example, *Arabidopsis thaliana* contains more than eight times as many intact *FBX* genes than the single-cell alga *Chlamydomonas reinhardtii*, and possibly ten times more than humans (~700 versus 83 and 69, respectively) (91).

From an evolutionary perspective, key questions remain: Why do plants encode so many CRL adaptors, particularly for SCF complexes, as compared with other eukaryotes? By what mechanism(s) did they expand during plant evolution? The simplest answer would be that the number of plant CRL adaptors directly correlates with the number of substrates, with both expanding in concert as plants evolved more sophisticated growth patterns, tissue types, and adaptive responses. The greater number of targets in land plants versus other eukaryotes could relate to their sessile growth habit, which for reasons not yet obvious might rely more heavily on posttranslational controls, or could reflect an innate defense mechanism used by land plants to ward off continuous attacks by pathogens and predators (65, 196). Comparisons among the *FBX* superfamilies in *C. reinhardtii*, the seedless plants *Physcometrilla patens* (bryophyte) and *Selaginella moellendorffii* (lycopod), and a number of monocots and eudicots are generally consistent with this view, as more primitive plants typically contain fewer *FBX* loci (91). Furthermore, one might imagine that the thousand or so CRLs in seed plants such as *Arabidopsis* and rice are matched with an equally large number of targets. Genetic analyses of a few well-characterized CRL adaptors in *Arabidopsis* support this one-to-one correspondence. For example, the six-member TIR/AFB1–5 FBX family likely controls the 27 AUX/IAA isoforms (142), the ETO1/EOL1/EOL2 BTB family targets the three type-II ACC synthases (23, 208), and the EBF1/EBF2 FBX pair recognizes the EIN3 and EIL1 transcriptional regulators (10).

However, recent phylogenetic studies show that the evolutionary patterns of plant CRLs are more complex than anticipated, especially for the SCF^{FBX} and BTB^{BTB} types. The first indication came from the analysis of BTB adaptors, which showed that the patterns of evolution were uneven among plant species and could be lineage specific. Whereas *Arabidopsis* contains 80 *BTB* genes with little or no obvious pseudogenes, rice contains 149 with evidence for at least 43 more pseudogenes (64, 65). This expansion in rice was not uniform across all BTB types but was explained by an increase in only one subfamily containing a Meprin and TRAF homology (MATH) recognition module, for which there are only 6 *MATH-BTB* genes in *Arabidopsis* but 76 in rice. Similar lineage-specific expansions of MATH-BTB proteins were evident in other monocots, indicating that monocots diversified this adaptor specifically after the monocot/eudicot split (65).

Subsequent studies with FBX adaptors detected lineage-specific evolutionary patterns

with even greater complexity than those for BTB adaptors (91, 217, 221). One surprise is that the *FBX* superfamily sizes vary considerably among plant species and appear unrelated to the growth habit (woody versus herbaceous), life cycle (annual versus perennial), or evolutionary history (91). For example, *Carica papaya* contains as few as 159 likely intact *FBX* loci, whereas *Arabidopsis lyrata* contains as many as 980 (**Table 1**). Total *FBX* gene numbers (intact and pseudogene) differ substantially even among closely related species. As illustrations, *Zea mays* and *Sorghum bicolor*, which split ~12 million years ago (Mya), differ by 400 total *FBX* loci, whereas *Arabidopsis* and *A. lyrata*, which split ~5 Mya, differ by 453 total *FBX* loci. Ortholog relationships showed that these extraordinary differences were caused by rapid lineage-specific gene gains (births) and losses (deaths), indicating that the *FBX* superfamily as a whole underwent considerable transformations during land-plant evolution. Similar to those observed for BTB proteins, birth/death rates were not commensurate among all FBX subtypes but showed striking enrichments and/or depletions of specific recognition modules in the various plant lineages (91).

An intriguing scenario is that the evolution of the plant *FBX*, *BTB*, and possibly the *DWD* superfamilies is directed by a genomic drift-type mechanism (91). This mechanism first proposed by Nei and coworkers (135, 136) postulates that, similar to genetic drift at the population level, neutral evolution randomly generates widely different gene family sizes among species. Although variations in sequence and copy number for drifting genes within a family may be largely inconsequential within populations, some may become fixed if they help acclimate the individuals to the new niches and habitats.

Like other gene families proposed to be affected by genomic drift [e.g., immunoglobulin and chemosensory receptor genes in vertebrates (135)], the plant *FBX* superfamily has all the hallmarks of genes evolving by this mechanism. First, the fact that the size of the *FBX* superfamily differs markedly and apparently at random across plant lineages implies that the birth/death history of the superfamily is for the most part arbitrary (91, 217, 221). Second, two subgroups of plant *FBX* loci can be detected (91). One smaller group encodes a highly conserved FBX collection that likely directs core and possibly ancient ubiquitylation events that are essential to most plants or even to eukaryotes in general. Another much larger group of *FBX* genes also exists that is under more relaxed or neutral selection, is highly lineage specific, and is often inconsequential to the plants. These more divergent genes share properties with obvious *FBX* pseudogenes, suggesting that they either control more species-specific functions or are nonfunctional and in the process of pseudogenization. In accord with the genomic-drift model, this more diverse collection could be generated at random by various events (e.g., polyploidy, segmental and tandem duplications, and retrotransposition) (91) to provide a dynamic reservoir of FBX proteins that can adjust ubiquitylation to improve plant fitness continually. Opportunities include creating new FBX adaptors with novel expression patterns, altered affinities for existing targets, and/or specificity toward new targets. If these birthed loci provide an adaptive advantage to the plant, they would become fixed. The lineage-specific ETP1/2 FBX proteins involved in EIN2 degradation may represent such a fixation (152). If not useful, the new *FBX* loci would eventually degenerate into pseudogenes or be lost. Such drift of *FBX* loci may also occur rapidly within populations given the extremely high rates of *FBX* polymorphisms that have been detected among *Arabidopsis* ecotypes (26).

If we assume that CRLs evolved by a genomic drift-type mechanism, several functional implications emerge. First, the birth/death histories of the *FBX* superfamily are more extreme than other large plant gene families (91), suggesting that genomic drift was applied to *FBX* genes and possibly those encoding other CRL adaptors with greater strength than most others. Coincidently, the SKP1 partners in SCF complexes may have also evolved by a similar drift in concert with FBX proteins, possibly

to preserve the same expression patterns and protein-protein interactions (108, 109). Second, given the high birth and death rates, most new *FBX* genes do not persist over a long time and rapidly become pseudogenes or are lost. Nonetheless, those that did acquire important roles, have clearly survived over the course of land-plant evolution. So, although genomic drift may have contributed to the initial emergence of new *FBX* loci in general, strong selection pressures would promote their long-term retention. Third, the one-to-one correspondence for CRL adaptors to substrates may be oversimplistic. For the core conserved adaptors, this may be true, but for the larger, more lineage-specific group, many may not have dedicated targets. This inert group may instead serve as seeds for continued CRL innovation through trial and error. Possibilities include the adaptive evolution of divergent CRLs for pathogen defense (65, 196) and self-recognition during plant reproduction (91).

CHALLENGES AHEAD

Even though much has been learned about plant CRLs since their discovery a decade ago (60, 71, 216), substantial challenges lie ahead in defining the full range of CRL E3 functions in plant biology. Clearly a combination of forward and reverse genetics, molecular interaction approaches, biochemistry, bioinformatics, and proteomics will be critical to reveal the functions of CRLs and link specific complexes to their cognate targets. Certainly, the possible scenario that only a subset of plant FBX adaptors (and maybe BTB and DWD as well) is biologically relevant with the rest subjected to genomic drift may reduce the task considerably by helping researchers focus on the conserved adaptors that actually direct physiologically relevant ubiquitylation events. Approximately 100 *Arabidopsis FBX* genes fit this criterion, thus concentrating genetic efforts to only a small fraction of the total *FBX* loci (91).

Unfortunately, each approach to studying CRLs and their targets has inherent limitations. Genetic approaches are complicated by functional redundancy. Whereas phenotypes can sometimes be elicited by removing the dominant gene in the subfamily [e.g., BTB^{ETO1} and ethylene synthesis (23, 208)], in other cases, inactivation of the entire set is required to observe a robust phenotypic defect [e.g., $SCF^{VIER1-4}$ (171) and $BTB^{LRB1/2}$ (M.J. Christians, D.J. Gingerich, R.D. Vierstra, unpublished)]. Even though the use of weak alleles can demonstrate the importance of core essential components (CULs, RBX1, SKP1s, DDB1, etc.) (63, 130, 159), their pleiotropic consequences are often too complicated to interpret because their composite phenotypes could reflect stabilization of hundreds of targets. Target predictions based on the phenotype of plants either missing or overexpressing individual adaptors (FBX, BTB, and DWD proteins) can be successful. For example, if a CRL E3 promotes degradation of the target, then hypomorphic or null mutants for the adaptor may resemble the phenotype of plants overexpressing the corresponding target(s) because the target(s) should be more stable and thus more abundant in the mutant background. Conversely, overexpressing CRL E3 may phenocopy mutations that disable or delete the target. Illustrations of this success includes linking $BTB^{ETO1/EOL1/EOL2}$ to type II ACS (23, 208), SCF^{COI1} to JAZ targets (22, 195), and $BTB^{LRB1/2}$ to phyB signaling (M.J. Christians, D.J. Gingerich, R.D. Vierstra, unpublished). Clearly, the more that is understood about plant development and physiology and their underpinning effectors, the better are the chances for success.

Biochemistry is emerging as a possible approach in defining E3/target pairs with the recent development of recombinant systems that can express functional CRL E3 complexes (146, 148, 160) and in vitro degradation systems that can mimic in planta turnover (141, 207). However, a major hurdle for many CRL E3s is the poor expression and insolubility of the individual components, which can sometimes be overcome by coexpression with other components [e.g., FBX and SKP1 (174, 192)]. Caution must also be taken to ensure that the in vitro

ubiquitylation reactions observed actually represent true E3/substrate specificities and not off-target events, given the high-energy intermediates involved in Ub addition. Interaction approaches can sometimes work (e.g., $SCF^{EBF1/2}$ and their targets EIN3 and EIL1) (61, 150), but if the target requires prior modification (e.g., phosphorylation or glycosylation) (145), it may be difficult to recapitulate in vitro or in heterologous systems such as yeast two-hybrid. Proteomic methods are now aiding in the identification of ubiquitylated proteins despite their low abundance and rapid turnover (125, 165). Fortunately, better conjugate purification methods (e.g., 85, 191) and improved mass spectrometers that should advance this approach have recently emerged. Unfortunately, connecting these proteins to specific E3s by combining proteomics with reverse genetics remains a challenge given the sheer number of Ub conjugates and ligases that plants normally contain. Hopefully, new methods such as the global protein stability assay developed with mammalian cells can be adapted to plant systems (222). Although many hurdles remain, we expect continued progress on defining CRL E3s and revealing their functions in plant biology.

DISCLOSURE STATEMENT

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