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Composition, Roles, and Regulation of Cullin-based Ubiquitin E3 Ligases

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Due to their sessile nature, plants depend on flexible regulatory systems that allow them to adequately regulate developmental and physiological processes in context with environmental cues. The ubiquitin proteasome pathway, which targets a great number of proteins for degradation, is cellular tool that provides the necessary flexibility to accomplish this task. Ubiquitin E3 ligases provide the needed specificity to the pathway by selectively binding to particular substrates and facilitating their ubiquitylation. The largest group of E3 ligases known in plants is represented by CULLIN-REALLY INTERESTING NEW GENE (RING) E3 ligases (CRLs). In recent years, a great amount of knowledge has been generated to reveal the critical roles of these enzymes across all aspects of plant life. This review provides an overview of the different classes of CRLs in plants, their specific complex compositions, the variety of biological processes they control, and the regulatory steps that can affect their activities.

INTRODUCTION

E3 ubiquitin ligases are ubiquitously present in eukaryotic organisms. First described in mammalian systems (Hershko and Ciechanover, 1982), they serve as central regulatory tools within the ubiquitin proteasome pathway where they provide specificity to the pathway and function as an interface between a ubiquitin (UBQ)-transfer cascade that consists of E1 UBQ activating and E2 UBQ conjugating enzymes, and subsequent 26S proteasome activities (Figure 1A). E3 ligases facilitate the ubiquitylation of substrate proteins to alter their function in various ways, most commonly by triggering substrate degradation (for a detailed description on the impact of ubiquitylation of substrates see the TAB chapter written by Judy Callis on ‘The Ubiquitination Machinery of the Ubiquitin System’).

E3 ligases can either be monomeric, or consist of multiple subunits, which through their interplay allow binding and ubiquitylation of specific substrates. A major class of multimeric E3 ligases that has been implicated in various developmental and physiological processes is represented by cullin-based E3 ligases. *Arabidopsis thaliana* encodes for six cullin-like proteins, CULLIN1 (CUL1: At4g02570), CULLIN2 (further referred to here as CUL1b: At1g02980 to avoid confusion with human Cul2), CULLIN3a (CUL3a: At1g25830), CULLIN3b (CUL3b: At1g69670), CULLIN4 (CUL4: At5g46210), and ANAPHASE PROMOTING COMPLEX2 (APC2: At2g04660). They range in size from 85 to 98 kDa, and are all characterized by a conserved cullin-region of around 200 amino acids in length. This cullin homology region comprises a 4-helix bundle, a winged helix-like domain, and an alpha- and beta-domain (Zheng et al., 2002b). Except for APC2, the C-terminal region also contains a modification site in which a single ubiquitin-related protein, NEURONAL PRECURSOR CELL EXPRESSED DEVELOPMENTALLY DOWNREGULATED8/ RELATED TO UBIQUITIN, NEDD8/RUB1; At1g31340 is covalently attached to the cullin. Two additional NEDD8/RUB1 homologs are expressed in Arabidopsis, RUB2; At2g35635, which is identical to RUB1 except for one amino acid change, and RUB3; At1g11980, which is 77.6% identical to the other two (Rao-Naik et al., 1998). Structural analyses revealed that the cullin serves as a scaffolding backbone of the E3 complex, which binds substrate adaptors through their N-terminal region, while their C-terminal domain is reserved for assembly with a RING (REALLY INTERESTING NEW GENE)-finger domain containing protein, RING BOX-1 (RBX1), which in turn binds a UBQ-carrying E2 enzyme (Schulman et al., 2000; Zheng et al., 2002b). Arabidopsis encodes for two of these RING-finger proteins, RBX1a/AtRBX1;1 (At5g20570) and RBX1b/AtRbx1;2 (At3g42830); however, only RBX1a appears to be significantly expressed (Gray et al., 2002 and Lechner et al., 2002). Cullins mainly vary within their N-terminal region and they are distinguished primarily by their affinity for specific classes of substrate adaptors. Based on these differences, four classes of cullin-RING (CRL) E3 ligases are currently recognized in plants (Figure 1B-E), which will be described and discussed in greater detail in the following sections.

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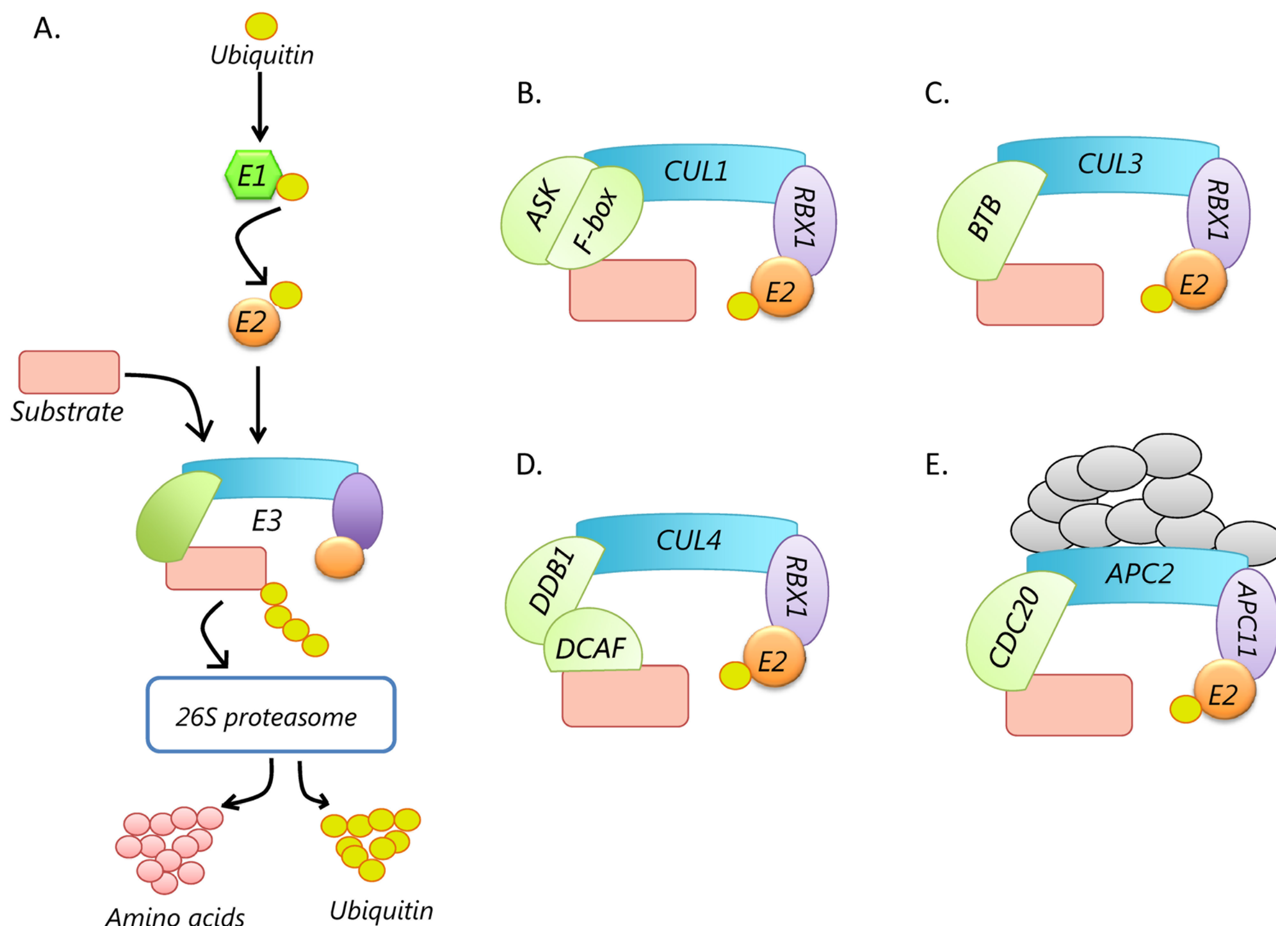

Figure 1. The Ubiquitin Proteasome Pathway and plant cullin-based E3 ligases.

(A) Model of the ubiquitin proteasome pathway and (B-E) the four cullin-based E3 ligases found within plants.

(B) SCF E3 ligases are composed of SKP1-like protein and an F-box containing protein that work together to bind to the CUL1 protein as well as facilitate targeting of specific substrates.

Additionally **(C)** CRL3 and **(D)** CRL4 complex utilize variety of adapter proteins in order to bind with the cullin in addition to the target substrate. CRL3 utilizing a single adaptor protein that contains a BTB/POZ domain while CRL4 complexes are similar to SCF complexes due to the use of two proteins, DDB1 and a DCAF protein, in order to target substrates and assembly with the cullin protein.

(E) The APC/C complex shows a greater diversity compare to the other classes of cullin-based E3 ligases due to the nature of this complex to utilize at least 11 subunits in addition to using a cullin- and an RBX1-like protein (APC2 and APC11 respectively) as the core scaffolding unit.

CUL1-BASED E3 LIGASES

Complex composition and structural organization of the different subunits

Probably the best-described and most diverse CRL family is represented by SCF (Skp1-Cdc53/CUL1-F-BOX) complexes. Crystallization studies of a human SCF complex showed that Skp1 and an F-box domain containing protein, Skp2, mediate interaction of substrates with the core SCF complex, consisting of Cul1 and Rbx1 (Schulman et al., 2000; Zheng et al., 2002b). The F-box protein serves as the primary substrate adaptor, and together with Skp1 mediates assembly of the substrate to the cullin. Arabidopsis encodes for two CUL1-like proteins, CUL1 and CUL1b. These proteins share 67% identity with each other and are around 86 kDa in size. However, because the biological role of CUL1b still remains elusive, we will focus the following on CUL1, which has been extensively described and appears to be the predominant component of SCF (SKP1-CULLIN-RBX1) complexes in Arabidopsis.

Crystallization studies on a human SCF complex have shown two main regions within Cul1. The N-terminal region contains three cullin-repeats. Each of these repeats are composed of a five-helix structural motif in which the helices C to E are organized as a three-helix bundle that interacts with helices A and B to form the cullin repeat (Zheng et al., 2002b). Specifically the first repeat is responsible for binding the substrate adaptor complex of Skp1

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and the F-box protein, while the other two repeats generate a rigid, arc-shaped structure that leads into the C-terminal domain (Zheng et al., 2002b). The C-terminal domain in turn contains the cullin-homology region and also mediates binding with the Rbx1/E2/UBQ complex. By the distinct structural arrangements of the quaternary SCF complex, the cullin brings the F-box and E2 moieties into close proximity of around 50A to facilitate transfer of UBQs to a substrate (Zheng et al., 2002b).

Skp1-like proteins contain an N-terminal domain fold of around 125 amino acids, which is similar to a BTB/POZ (Broad complex, Tramtrack, Bric-a-brac/Pox virus and Zinc finger) domain (Schulman et al., 2000), and is used by Skp1 to interact with the first cullin repeat. The F-box protein interacts with both Skp1 and the first cullin repeat, and uses primarily its F-box motif, a three α-helical fold, which was first identified in cyclin F (Schulman et al., 2000).

While only two CUL1-like and two RBX1 proteins are encoded in Arabidopsis, a much larger number of S-PHASE KINASE-ASSOCIATED PROTEIN 1 (SKP1)-like and F-box proteins exist. Arabidopsis encodes for 21 SKP1-like or ARABIDOPSIS SKIP (ASK)-like proteins (Risseeuw et al., 2003), which can be grouped into two major clades, with ASK1 (At1g10940) to 19 (At2g03160) representing one clade while ASK20 (At2g45950) and ASK21 (At3g61415) comprising the other. This strong clustering of ASK proteins is a likely indicator of a common ancestor (Risseeuw et al., 2003; Kong et al., 2007). Of the 21 known *ASK* genes, *ASK9* (At3g21850) and *ASK15* (At3g25650) are likely to be pseudogenes based on premature stop codons. All other *ASKs* are expressed and are prospective subunits within an SCF complex (Risseeuw et al., 2003). Association with CUL1 has been demonstrated for many of these proteins (Risseeuw et al., 2003).

In addition to these core subunits, plant SCF complexes can also assemble with the accessory protein SUPPRESSOR OF THE G2 ALLELE OF SKP1 (SGT1). Arabidopsis encodes for two closely related SGT1 proteins, SGT1a (At4g23570) and SGT1b (At4g11260), which mainly have been brought into context with early plant defense responses, but also with auxin signal transduction (Austin et al., 2002; Azevedo et al., 2002; Gray et al., 2003; Walsh et al., 2006; Wang et al., 2010; Anand et al., 2012). However, we will focus in the following on the SCF core subunits.

By far the greatest diversity is found in the F-box protein superfamily, which in *A. thaliana* consists of 694 predicted members, and up to 1350 predicted members in the closely related species *A. lyrata* (Gagne et al., 2002; Hua et al., 2011). Besides the F-box domain, these proteins frequently contain additional protein-protein interaction motifs such as LRR (leucine-rich), KELCH, or WD40 repeats, to facilitate binding of specific substrates (Gagne et al., 2002; Hua et al., 2011). A recent large scale, yeast-2-hybrid interaction study tested assembly of 341 F-box proteins with 19 ASK family members. The work showed that seven ASK proteins (ASK1-3 and ASK11-14) were each able to interact with more than 40 different F-box proteins, while others, like ASK4, ASK5, and ASK6, showed interactions with less than five (Kuroda et al., 2012). This may be indicative of a situation where only a subset of ASK proteins significantly participate in SCF activities. Also of interest is the large number of F-box proteins, 201 in total, that did not interact with any ASK proteins in these studies, suggesting that secondary modifications, such as a phosphorylation, may be required to trigger assembly into an SCF complex (Kuroda et al., 2012).

SCF function in higher plants

Based on the great number of F-box proteins, one can expect that SCF complexes participate in many cellular and developmental processes in plants. This is supported by the wide range of phenotypes described for hypomorphic and null mutants of *CUL1*. While complete loss of the cullin is embryo-lethal and causes abortion of embryogenesis at the early globular stage, hypomorphic mutants show pleiotropic defects related to lateral organ initiation, flower development, photomorphogenesis, and auxin and jasmonate signaling, as well as the circadian clock (Shen et al., 2002; Hellmann et al., 2003; Quint et al., 2005; Ren et al., 2005; Moon et al., 2007; Harmon et al., 2008; Llorente et al., 2008; Gilkerson et al., 2009). Likewise, loss of ASK1 and ASK2, which interact with a broad range of F-box proteins, causes defects in early seedling establishment, root and flower development, aberrant male meiotic progression and male sterility, as well as embryogenesis (Zhao et al., 1999; Zhao et al., 2003; Liu et al., 2004; Wang and Yang, 2006; Li et al., 2012).

Consequently, the best approach to dissect the diverse roles of SCF complexes is given by studying the function of subgroups of F-box proteins and their individual members. In the following we will focus on some of the major processes, namely phytohormone and light signaling, cell cycle regulation, and pathogen-related processes, which have been identified in recent years to rely on specific F-box proteins. Many of the depicted examples also point out the importance of modifications to the F-box or substrate proteins to promote formation of F-box/substrate complex assemblies. An overview of processes linked to the SCF complex is shown in Figure 2, and a summary of the F-box proteins discussed below is presented in Table 1.

Phytohormone regulation

One of the most prominent functions of F-box proteins is their participation in all of the phytohormone signal transduction pathways, where they have been demonstrated as central regulators, and in some cases hormone receptors in these pathways (Dill et al., 2004; Gagne et al., 2004; Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Thines et al., 2007; Gou et al., 2009; Nelson et al., 2011; Zheng et al., 2011; Peng et al., 2012; Shen et al., 2012; Kim et al., 2013; Van der Does et al., 2013). A majority of identified targets are transcription factors, and in many cases the associated phytohormone serves as a direct regulator that is required to trigger assembly of the F-box protein with their substrates.

TRANSPORT INHIBITOR RESPONSE 1 (TIR1; At3g62980) provided one of the first examples of an F-box protein in phytohormone signal transduction, specifically in auxin signal transduction, as part of an SCF^{TIR1} E3 ligase complex (Ruegger et al., 1998; Gray et al., 1999; Gray et al., 2001). TIR1 contains an LRR motif that comprises 18 repeats that are critical for substrate binding (Ruegger et al., 1998; Tan et al., 2007). However, to efficiently interact with substrates, TIR1 needs to first bind auxin (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Tan et al., 2007; Calderon Villalobos et al., 2012). Crystal structure analysis of a TIR1/ASK1 complex showed that the dimer has a mushroom-shaped organization with the LRR domain of TIR1 forming the

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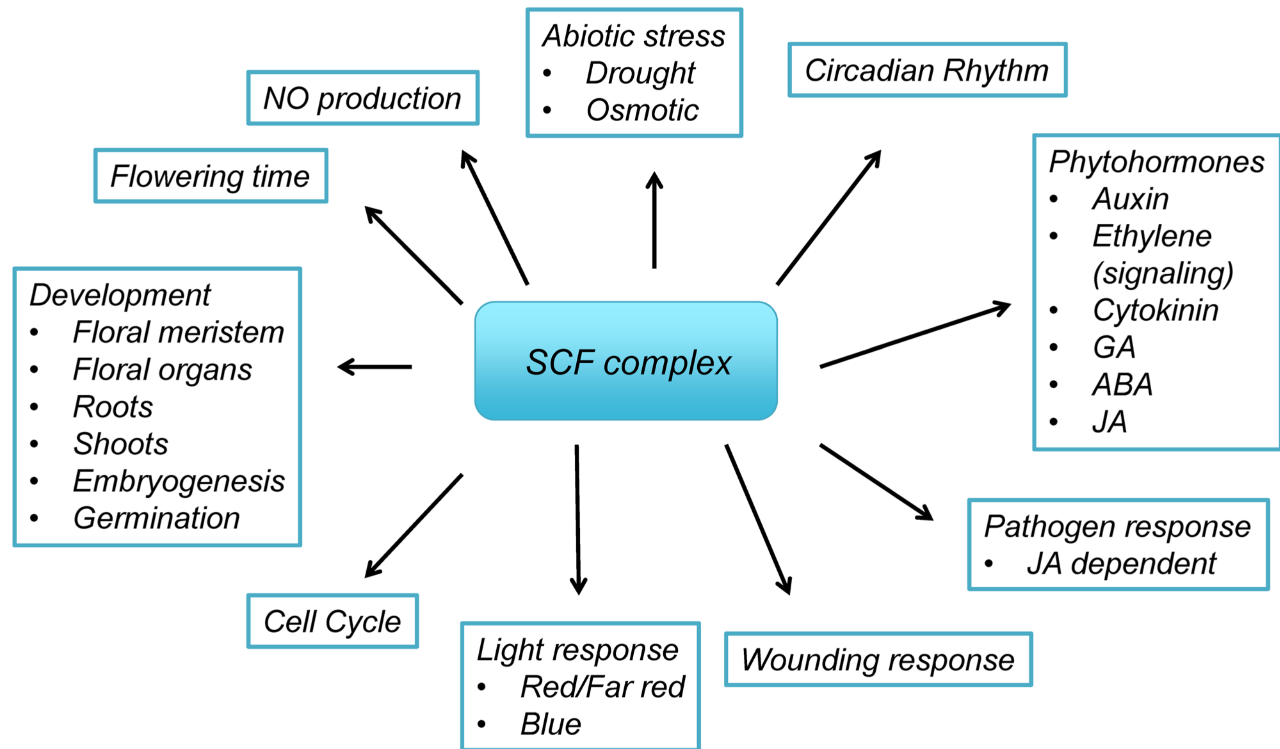

Figure 2. SCF complexes participate in a wide variety of plant processes.

cap, while the stem consists of TIR1's F-box motif and ASK1 (Tan et al., 2007). The LRR domain of TIR1 folds into a twisted horseshoe-shaped solenoid structure providing a binding pocket for auxin (Tan et al., 2007). Binding of auxin stimulates assembly with Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) proteins and their subsequent ubiquitylation and degradation (Gray et al., 2001; Kepinski and Leyser, 2004). TIR1 therefore acts as a receptor in auxin signal transduction, which was a novel finding for an F-box protein (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005).

Analysis of the TIR1/ASK1 crystal structure also revealed the presence of inositol hexakisphosphate (InsP6) in the center of TIR1's LRR fold, near the site designated for auxin binding (Tan et al., 2007). Targeted mutagenesis of residues participating in InsP6 binding showed that this disrupts not only the ability of TIR1 to interact with the Aux/IAA protein IAA7 (At3g23050), but also ASK1 (Calderon Villalobos et al., 2012). These findings emphasize that InsP6 has a critical role for the structural organization of TIR1 and its function as auxin receptor.

Aux/IAA proteins function as negative regulators in auxin signal transduction by repressing the activity of AUXIN RESPONSE FACTOR (ARF) transcription factors (Maraschin Fdos et al., 2009). Consequently, Aux/IAA protein degradation liberates ARFs, allowing auxin responsive gene expression (Maraschin Fdos et al., 2009). This auxin induced gene expression also upregulates *Aux/IAA* gene expression, generating a negative feedback loop in auxin signal transduction (Lee et al., 2009a).

Aux/IAA proteins contain four domains (DI to IV) of which DI can interact with the transcriptional repressors TOPLESS (TPL) (Szemenyei et al., 2008), while DII contains the degron motif, and is required for interaction with TIR1 (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). DIII and DIV facilitate oligomerization with Aux/IAA and ARF proteins (Ulmasov et al., 1997; Ouellet et al., 2001; Nanao et al. 2014). Remarkably, studies on IAA7 using mutations in DII showed that IAA7 assembly with TIR1 is required for efficient auxin binding, demonstrating that Aux/IAA and TIR1 actually function as co-receptors for the phytohormone (Calderon Villalobos et al., 2012).

TIR1 belongs to a small TIR1/AUXIN-SIGNALING F BOX (AFB) family that comprises six members in Arabidopsis, all of which have the ability to bind auxin-like compounds (Dharmasiri et al., 2005b; Parry et al., 2009; Greenham et al., 2011). Due to functional overlap, *tir1* null mutants display no major developmental defects. However, loss of the four closest *TIR1/AFB* members, *TIR1*, *AFB1 (At4g03190)*, *AFB2 (At3g26810)* and *AFB3 (At1g12820)*, results in severely affected development, most prominently expressed by reduced root and shoot growth as well as aberrant embryo development (Dharmasiri et al., 2005b). Interestingly, recent findings suggest that AFB4 (At4g24390), and potentially also AFB5 (At5g49980), function as negative regulators in auxin signal transduction based on the auxin-hypersensitivity of an *afb4-1* null mutant (Greenham et al., 2011). How AFB4 functions precisely to negatively affect auxin responses still remains elusive. Besides auxin, the activity of TIR1, AFB2 and AFB3, but not AFB1, is also regulated by the micro RNA miR393 (At2g39885) (Navarro et al., 2006; Parry et al., 2009). The regulation of the three TIR1/AFB members through this miR393 is

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Table 1. SCF substrate adapters described in this chapter

Protein Name	AGI Number	Proposed Function	References
COI1	At2g39940	JA signaling, pathogen response, wounding, pollen fertility	Xie et al 1998; Feys et al 1994; Thines et al 2007
DOR	At2g31470	Drought tolerance, ABA-stomatal response	Zhang et al 2008b
EBF1 EBF2	At2g25490 At5g25350	Ethylene signaling,	Guo & Ecker 2003; Potuschak et al 2003; Binder et al 2007
EDL3	At3g63030	ABA signaling, seed germination, flowering time, root development, osmotic stress	Koops et al 2011
EID1	At4g02440	Photomorphogenesis, skotomorphogenesis; far-red response	Dieterle et al 2001; Marroco et al 2006
ETP1 ETP2	At3g18980 At3g18910	Ethylene signaling	Alonso et al 1999; Qiao et al 2009
FBL17	At3g54650	Cell cycle regulation, gametogenesis	Gusti et al 2009; Kim et al 2008
FKF1	At1g68050	Blue light response, flowering time, photomorphogenesis,	Sawa et al 2007; Ito et al 2012
KMD -KMD1 -KMD2 -KMD3 -KMD4	 At1g80440 At1g15670 At2g44130 At3g59940	Cytokinin signaling	Kim et al 2013; Mueller & Sheen 2007
LKP2	At2g18915	Blue light response, flowering time, photomorphogenesis,	Ito et al 2012
MAX2	At2g42620	ABA signaling, ABA-stomatal response, drought tolerance, osmotic stress, strigolactone signaling, lateral shoot branching	Bu et al 2014; Stirnberg et al 2007
SGT1a SGT1b	At4g23570 At4g11260	Auxin signaling, pathogen response	Austin et al 2003; Azevedo et al 2002; Gray et al 2003
SKP2a	At1g21410	Cell cycle regulation, auxin,	del Pozo et al 2002a; Jurando et al 2010
SKP2b	At1g77000	Cell cycle regulation, kinase activity	del Pozo et al 2002a; Ren et al 2008
SLY1 SLY2	At4g24210 At5g48170	GA signaling	Strader et al 2004; Ariizumi et al 2011
TIR1/AFB -TIR1 -AFB1 -AFB2 -AFB3 -AFB4 -AFB5	 At3g62980 At4g03190 At3g26810 At1g12820 At4g24390 At5g49980	Auxin signaling, root development, shoot growth, embryogenesis, drought tolerance, nitric oxide production	Gray et al 1999; Dharmasirii et al 2005b; Gray et al 2001; Greenham et al 2011
UFO	At1g30950	Flower organogenesis, floral meristem development	Levin & Meyerowitz 1995; Chae et al 2008;
ZTL	At5g57360	Blue light response, flowering time, photomorphogenesis, circadian rhythm,	Kiba et al 2007; Ito et al 2012

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critical for proper auxin signal transduction, the establishment of the root architecture, and drought stress responses (Chen et al., 2011; Si-Ammour et al., 2011; Windels and Vazquez, 2011; Chen et al., 2012).

An additional posttranscriptional mechanism of regulating SCF^{TIR1} activity has been reported in context with nitric oxide (NO) (Terrile et al., 2012). NO is a gaseous molecule that affects a range of developmental processes and stress responses, and can function as a second messenger in cells (recently reviewed by Yu et al., 2014). Auxin induces NO production, and NO in turn further stimulates binding of TIR1 to Aux/IAA proteins, which is connected with S-nitrosylation of a cysteine residue of TIR1 (Terrile et al., 2012). It is currently unclear whether this secondary modification also applies to other TIR1/AFB members, but the finding further demonstrates the complexity of regulatory tools the cells has in place to control SCF$^{TIR1/AFB}$ activities.

The F-box protein that is most closely related to the TIR1/AFB family is CORONATINE-INSENSITIVE PROTEIN 1 (COI1; At2g39940) (Xie et al., 1998). Similar to TIR1 it contains a 16 repeat LRR motif that is critical for substrate binding. As its name implies, COI1 was originally identified in a screen for mutants resistant towards the phytotoxin coronatine, a jasmonic acid-isoleucine (JA-Ile) analog (Feys et al., 1994). As it turned out however, COI1 represents the central regulator in jasmonic acid (JA) signal transduction (Xie et al., 1998; Devoto et al., 2002). Loss of COI1 leads to reduced wound and defense responses and male sterility as they lack viable pollen (Xie et al., 1998).

Molecular characterization revealed that COI1 functions in a similar fashion to TIR1, as a phytohormone receptor, by binding specifically to JA-Ile to trigger degradation of transcriptional repressors (Thines et al., 2007; Yan et al., 2009; Sheard et al., 2010; Pauwels and Goossens, 2011). Targets of the SCF^{COI1} complex are JASMONATE ZIM-DOMAIN (JAZ) proteins (Thines et al., 2007; Yan et al., 2009). They comprise a small gene family with 10 members in Arabidopsis, and their expression is rapidly induced by JA treatment (Thines et al., 2007). Of note is that JAZ binding by COI1 requires inositol pentakisphosphate (InsP5), which binds to both, COI1 and JAZ (Sheard et al., 2010).

JAZ proteins do not contain a DNA binding domain. They instead block activity of specific transcription factors such as basic helix-loop-helix subgroup IIIe transcription factors (MYC2 (At1g32640), MYC3 (At5g46760), MYC4 (At4g17880)) or members of the MYB transcription factor family (Qi et al., 2011; Song et al., 2011; Shyu et al., 2012; Song et al., 2013) either directly or in concert with co-repressors. The JA triggered degradation of JAZ proteins relieves repression of MYC/MYB transcription factors, and allows JA-dependent up-regulation of responsive genes. Similar to the Aux/IAAs, JA-induction of *JAZ* genes creates a feedback loop that allows the cell to subsequently attenuate the JA response.

Recently, involvement of SCF complexes in cytokinin signal transduction has also been demonstrated. Here a group of KISS ME DEADLY (KMD) F-box proteins has been identified that function within an SCF complex to target B-type ARABIDOPSIS RESPONSE REGULATORS (Type-B ARR) proteins (Muller and Sheen, 2007; Kim et al., 2013). Type-B ARRs are activated by a phosphate-transfer chain and serve as the main, positive, cytokinin response regulators at the transcriptional level (Kushwah et al., 2011). In addition to their N-terminal F-box motif, the four KMD family members have a KELCH repeat domain with a β-propeller structure that likely mediates interaction with B-type ARRs. KMD proteins function as negative regulators in cytokinin signal transduction by controlling stability of B-type ARRs. This has been demonstrated by genetic and biochemical approaches (Kim et al., 2013), and in addition, overexpression of KMD1 (At1g80440) leads to significant cytokinin insensitivity (Kim et al., 2013).

Ethylene signal transduction also involves steps in which SCF complexes and the 26S proteasome play a role. The hormone widely affects physiological and developmental processes in plants. Most of the proteins participating in ethylene perception and signal transduction have been identified by screening for mutants with an aberrant triple response, which in WT comprises shortened hypocotyl and root along with an exaggerated apical hook in seedlings germinated in the dark (Johnson and Ecker, 1998). In contrast to TIR1 or COI1, which are positive response mediators, F-box proteins identified to participate in the ethylene response pathway, function as negative regulatory proteins. For example, in the absence of ethylene, EIN3-BINDING F-BOX 1 (EBF1; At2g25490) and EIN3-BINDING F-BOX 2 (EBF2; At5g25350) mediate the ubiquitylation and degradation of the transcription factor ETHYLENE INSENSITIVE 3 (EIN3; At3g20770)) (Guo and Ecker, 2003; Potuschak et al., 2003; Binder et al., 2007). EIN3 represents a central, positive mediator in ethylene signal transduction, and its degradation, initiated through SCF$^{EBF1/EBF2}$ E3 ligases, prevents an ethylene response. Binding of ethylene to an ethylene receptor inactivates the corresponding SCF$^{EBF1/EBF2}$ complexes, and the consequential stabilization of EIN3 allows an ethylene-induced transcriptional response (Guo and Ecker, 2003; Potuschak et al., 2003; Binder et al., 2007). As described for JAZ and Aux/IAA proteins, ethylene signal transduction also involves a negative feedback loop, since one of the targets of the EIN3 transcription factor is the *EBF2* gene (Konishi and Yanagisawa, 2008), indicating a common regulatory scheme in signal transduction pathways that involve SCF activities.

Upstream of EBF1/EBF2 is factor ETHYLENE INSENSITIVE 2 (EIN2; At5g03280), an ER-localized transmembrane protein with similarities to NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN (NRAMP) transporters (Alonso et al., 1999). EIN2 contains a cytosolic extension that is cleaved off in response to ethylene to allow passage into the nucleus. This translocalization of the EIN2 cytosolic tail causes the proteasomal degradation of EBF1 and EBF2 by yet unknown mechanisms (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). In addition, EIN2 itself is the target of SCF complex activities that employ the F-box proteins EIN2 TARGETING PROTEIN1 (ETP1; At3g18980) and EIN2 TARGETING PROTEIN2 (ETP2; At3g18910) as substrate adaptors in the absence of ethylene (Alonso et al., 1999; Qiao et al., 2009). At least one of the ethylene receptors, ETHYLENE RESPONSE 2 (ETR2; At3g23150), is also degraded in a 26S proteasome-dependent manner, but the corresponding E3 ligase has not been identified yet (Chen et al., 2007).

In the case of gibberellic acid (GA) signaling, GA can bind to one of three different receptors named GA-INSENSITIVE DWARF1 (GID1a; At3g05120, GID1b; At3g63010, GID1c; At5g27320), (Griffiths et al., 2006) that are most likely nuclear localized. Binding of GA activates these receptors to form a complex with DELLA proteins (Griffiths et al., 2006), which carry a conserved N-terminal DELLA amino acid sequence. They form a

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small protein family of five members in Arabidopsis that function as transcriptional repressors of GA response (Dill et al., 2004; Ariizumi et al., 2011; Ariizumi and Steber, 2011). DELLA proteins also have an additional VHYNP motif in their N-terminal region that, together with the DELLA motif, is required to mediate interaction with GID1 receptors (Griffiths et al., 2006). Formation of the GID1/DELLA complex in turn is required to promote association with either one of two F-box proteins, (SLY1; At4g24210) and SLEEPY2/SNEEZY (SLY2/SNE; At5g48170), which result in DELLA protein ubiquitylation by the SCF^{SLY} complex (Strader et al., 2004; Ariizumi et al., 2011). Besides their N-terminal F-box motif, SLY1 and SLY2, do not contain any obvious additional folds, but their C-terminal region is required to recognize and bind to DELLA proteins (McGinnis et al., 2003).

Several F-box proteins have been described to mediate responses of the classic GA antagonist, abscisic acid (ABA) (Zhang et al., 2008b; Koops et al., 2011; Peng et al., 2012; Shen et al., 2012; Bu et al., 2014). One of the earliest reports describes the function of DROUGHT TOLERANCE REPRESSOR/S-LOCUS F-BOX-LIKE35 (DOR/AtSFL35; At2g31470) (Zhang et al., 2008b), which was identified in a screen for T-DNA insertion mutants that were hypersensitive to drought stress, classifying the protein as a negative regulatory factor. DOR is part of the Class C *AtSFL* gene family of F-box proteins that have been brought into context with self-incompatibility (Wang et al., 2004b). However, *DOR* itself is predominantly expressed in the stomata, and its overexpression results in drought hypersensitivity, which correlates with aberrant stomata closure (Zhang et al., 2008b). In agreement with this stomata-specific expression of *DOR*, null mutants do not display changes in other processes, such as germination or flowering, that are associated with ABA. *DOR* expression is negatively regulated by ABA and drought stress, potentially as part of a negative feedback loop (Zhang et al., 2008b). As of now specific substrates for DOR are unknown, and it appears to be that the protein assembles with only a very limited number of SCF complexes since out of 17 tested ASK proteins, it exclusively interacted with ASK14 (Zhang et al., 2008b).

EMPFINDLICHER IM DUNKELROTEN LICHT1-LIKE PROTEIN3 (EDL3; At3g63030) is a second F-box protein that is involved in ABA signaling (Koops et al., 2011). Genetic studies on overexpressors and conditional knockout mutants demonstrated that EDL3 participates in a wide range of ABA-affected developmental processes such as seed germination, flowering control, or root growth (Koops et al., 2011). In contrast to DOR, it is a positive mediator of ABA response since overexpressor plants are hypersensitive to ABA in germination assays, while loss-of-function results in ABA resistance (Koops et al., 2011). In addition, *EDL3* expression is inducible by ABA and a variety of osmotic stress conditions, and it can interact with a variety of ASK proteins (Koops et al., 2011).

A third example for an F-box protein that is involved in ABA signaling is MORE AXILLARY GROWTH2 (MAX2; At2g42620). Like EDL3, MAX2 appears to be a positive ABA response mediator since loss-of-function plants are less sensitive to the phytohormone. They show reduced ABA-dependent stomata responsiveness and aberrant expression of ABA-responsive genes, and are hypersensitive to drought and other osmotic stresses (Bu et al., 2014). In germinating seeds, MAX2 appears to function as a mediator in multiple phytohormone pathways, as it positively affects expression of genes causing GA inactivation, ABA biosynthesis and catabolism. Additionally, MAX2 also appears to have a role in auxin distribution indicated by increased sensitivity of *max2* mutants towards the auxin transport inhibitor 1-N-Naphthylphthalamic acid (Shen et al., 2012).

It will be interesting to understand how the plant coordinates regulation of these F-box proteins, especially, since EDL3 and MAX2 are positive mediators of an ABA response, while DOR is a negative response regulator. Several possibilities include specific feedback loops, as well as their individual tissue-specific, stress and ABA-dependent gene expression patterns. In addition, MAX2 was originally described in context with strigolactones as a negative regulator in lateral shoot branching (Stirnberg et al., 2007; Nelson et al., 2011), positioning this F-box protein in an even more central role within phytohormone signaling pathways.

SCF complexes in light, circadian clock regulation, and flowering control

Light represents the essential energy source for photosynthesis, but it is also a vital environmental cue that affects physiological and developmental programs. Plants can distinguish certain light qualities as well as quantities, and these external signals are perceived, measured and integrated by specific photoreceptors and downstream signal transduction pathways. Red and far-red light are perceived by phytochrome (phy) photoreceptors that primarily affect processes related to photomorphogenesis (Chen and Chory, 2011). The EMPFINDLICHER IM DUNKELROTEN LICHT1 (EID1; At4g02440) F-box protein was identified within a screen for mutants exhibiting hypersensitivity towards far-red light exposure. The corresponding mutants undergo a switch from skoto- to photomorphogenesis (open cotyledons and a reduced hypocotyl growth) under weak far-red light, conditions at which wild type plants remain etiolated (Dieterle et al., 2001). This far-red light hypersensitivity characterized EID1 as a negative regulator in a phyA-dependent pathway (Dieterle et al., 2001; Marrocco et al., 2006; Muller et al., 2009), although the precise interplay between the two proteins remains elusive. EID1 can interact in yeast-2-hybrid and bimolecular fluorescence complementation experiments with a broad variety of ASK family members (Marrocco et al., 2006), but substrates have not been identified for EID1 so far that would significantly clarify its function in plants.

Another interesting group of F-box proteins that participate in blue light-dependent regulation, the circadian clock, photomorphogenesis, and flowering time control is the ZEITLUPE/FLAVIN-BINDING, KELCH REPEAT, F-BOX1/LOV KELCH PROTEIN2 (ZTL(At5g57360)/FKF1(At1g68050)/LKP2(At2g18915)) protein family (Han et al., 2004; Baudry et al., 2010; Takase et al., 2011). These proteins contain a LOV (Light, Oxygen, or Voltage) domain in their N-terminal region, followed by an F-box motif, and six KELCH repeats in their C-terminal region (Ito et al., 2012). All three members of this family serve as photoreceptors based on their LOV domain, which binds to a flavin mononucleotide (FMN) cofactor and undergoes conformational changes when exposed to blue light (Kasahara et al., 2002; Kasahara et al., 2010). In addition, the domain also serves as a protein-protein interaction fold that mediates homo- and heterodimerization among the dif-

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ferent family members, as well as with other proteins (Yasuhara et al., 2004).

The second important role of the ZTL/FKF1/LKP2 family is the control of the circadian clock (Baudry et al., 2010), in which ZTL appears to play a predominant role. A co-dominate *ztl-1* mutant was originally identified in a non-invasive screen for plants with aberrant photoperiod phenotypes (Somers et al., 2000). ZTL controls the stability of at least two core clock proteins, TIMING OF CAB EXPRESSION1 (TOC1; At5g61380) and PSEUDO RESPONSE REGULATOR5 (PRR5; At5g24470) (Mas et al., 2003; Kiba et al., 2007). For TOC1, it has been demonstrated in yeast-2-hybrid experiments that the LOV domain mediates assembly between ZTL and the core clock protein (Mas et al., 2003). For PRR5, strong evidence suggests that the LOV domain plays a critical regulatory role in mediating its blue-light dependent and 26S proteasome-dependent degradation in context with the circadian clock and in early photomorphogenesis (Kiba et al., 2007).

The role of FKF1 as a positive regulator in flowering time control is well established. Mediated by its blue-light excited LOV domain, it forms a complex with GI (GIGANTEA; At1g22770) and CDF1 at the promoter of *CONSTANS (CO; At5g15840*), a key regulator in controlling expression of the florigen *FLOWERING LOCUS T (FT; At1g65480*) (Sawa et al., 2007; Song et al., 2012). *CO* expression is negatively regulated by CDF1, and the activation of FKF1 through blue-light, in complex with GI, leads to the proteasomal degradation of CDF1 and induction of *CO* expression. Evidence based on chromosomal immunoprecipation (ChIP) data point out that FKF1 may also be directly involved in controlling *FT* expression as it can be found associated with the *FT* promoter (Sawa and Kay, 2011). Although ZTL and LKP2 function in flowering time control as well, their role is less well understood and appears to be more complex than that of FKF1. As this has been extensively reviewed recently, we refer here to Ito and co-workers for further reading (Ito et al., 2012). As described for the phytohormone-dependent signal transduction pathways, the light regulated ZTL/FKF1/LKP2 family is another excellent example of how dependent the corresponding SCF E3 ligases are on defined regulatory signals to control their activities.

Besides controlling the flowering time, SCF complexes have also been implicated in flower organ development. A key F-box protein in this process is UNUSUAL FLORAL ORGANS (UFO; At1g30950), which is critical for normal development of both the floral meristem and floral organs. Consequently, *ufo* mutants are characterized by reduced numbers, or even a complete loss, of stamen and petals (Levin and Meyerowitz, 1995; Hepworth et al., 2006; Risseeuw et al., 2013). UFO interacts with ASK1 and ASK2 proteins using its N-terminal F-box domain while the C-terminal region is employed to bind LEAFY (LFY; At5g61850) (Samach et al., 1999; Zhao et al., 2001). LFY is a master regulatory transcription factor that controls meristem identity and floral organ development by positively regulating expression of floral homeotic genes (Huala and Sussex, 1992; Schmid et al., 2003). Among these is the B-class MADS (MCM Agamous Deficiens SRF)-box transcription factor *APETALA3 (AP3; At3g543403*), which is directly controlled by LFY. The interaction between UFO and LFY is critical to bring LFY to the *AP3* promoter (Chae et al., 2008). LFY is also a target of SCF^{UFO} dependent ubiquitylation, and although proteasomal activity is required for full expression of *AP3*, it remains open whether LFY is the primary target of SCF^{UFO} activities (Chae et al., 2008). Since LFY is a positive modulator of *AP3* expression it appears to be likely that repressors of *AP3* expression are targeted first by SCF^{UFO} to become degraded by the 26S proteasome.

Cell cycle regulation by SCF complexes

A connection of plant SCF complexes with cell cycle processes had already been found comparably early when, in 2002, CUL1 was demonstrated to co-localize with the mitotic spindle in metaphase (Shen et al., 2002). Mainly work on the F-box proteins SKP2a (At1g21410), SKP2b (At1g77000), and F-BOX-LIKE 17 (FBL17; At3g54650), has provided more specific insights on how SCF complexes participate in cell cycle control.

In Arabidopsis SKP2a and SKP2b are closely related and contain a LRR motif for substrate binding in their C-terminal region (del Pozo et al., 2002a). Interaction of SKP2a with ASK1, ASK2 and weakly with ASK18 has been demonstrated along with binding to and mediation of ubiquitylation of the transcription factors E2Fc (At1g47870) and DPB (At5g03415) (del Pozo et al., 2002a; Jurado et al., 2008). E2Fc and DPB belong to small gene families with six and two members in Arabidopsis, respectively (Shen, 2002; Del Pozo and Manzano, 2013). They are part of the RBR/E2F/DP pathway that functions as a checkpoint control in cell cycle progression from G1 to S phase (Kuwabara and Gruissem, 2014). E2F and DPB form heterodimers, and their activities are repressed by RETINOBLASTOMA (RBR; At3g12280), while phosphorylation of RBR by CYCLIN-DEPENDENT KINASE (CDKs) releases RBR from the E2F/DPB heterodimers and induces gene transcription (Uemukai et al., 2005; del Pozo et al., 2006; Magyar et al., 2012). E2Fc, like RBR, likely functions as a transcriptional repressor and negative regulator of cell cycle progression (del Pozo et al., 2002a; del Pozo et al., 2006). In addition *in vitro* phosphorylation studies with CYCLIN-DEPENDENT KINASEA;1/CYCLIN2;1 (CDKA;1(At3g48750)/CYCD2;1(At2g22490)) demonstrated that E2Fc is also a target of CDKs, and phosphorylation is critical to mediate interaction with SKP2a (del Pozo et al., 2002a).

SKP2a itself is a target of the 26S proteasome (Jurado et al., 2008), and, like TIR1 and AFB proteins, contains an auxin-binding site, which promotes SKP2a-DPB interaction, as well as degradation of the transcription factor (Jurado et al., 2010). Binding of the phytohormone by SKP2a has been suggested as a mechanism to directly control and induce cell proliferation by auxin.

Also of interest is that SKP2b has been implicated in controlling stability of the cyclin-dependent kinase inhibitor KIP-RELATED PROTEIN 1 (KRP1; At2g23430) (Ren et al., 2008), which assembles with and represses the kinase activity of the CDKA;1/CYCD2;1 complex. SKP2b-dependent degradation of KRP1 likely activates CDKA;1 and promotes phosphorylation of its substrates. One may expect that this in turn can trigger SKP2a-mediated degradation of E2Fc and DPB.

The F-box protein FBL17 also participates in G1-S transition, but appears to have a more specialized function in cell cycle regulation during microgametogenesis, since loss of FBL17 specifically affects the second pollen mitosis and results in bicellular pollen (Gusti et al., 2009). FBL17 is an LRR-type F-box protein

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that predominantly interacts with ASK11 in yeast-2-hybrid studies (Gusti et al., 2009), and targets KRP6 (At3g19150) and KRP7 (At1g49620) for degradation (Kim et al., 2008). These two closely related cyclin-dependent kinase inhibitors are required for gametogenesis, and can interact with FBL17 (Kim et al., 2008; Gusti et al., 2009). However, further studies on FBL17 activities and the roles of KRP6 and KRP7 are needed to better understand their precise impact on cell cycle regulation.

Overall the work on F-box proteins discussed in this section has so far found that plant SCF complexes primarily participate in the G1 to S-phase transition, but not in other steps of the cell cycle.

SCF complexes hijacked by pathogens

The great diversity of different F-box proteins that target a vast number of substrates in a very flexible manner also provides a good anchor point for pathogens to take advantage of this pathway. Below, three examples are briefly described that are likely representative of a more broader plant-pathogen interplay yet to be elucidated through future research.

The first example is given by the F-box protein CELL CYCLE LINK (CLINK), for which the corresponding gene is widely abundant in the ssDNA genomes of nanoviruses (Lageix et al., 2007). Its C-terminal region contains an LxCxE motif that mediates binding to RBR (Aronson et al., 2000; Lageix et al., 2007), while it's N-terminal F-box motif allows assembly with ASK1 (Aronson et al., 2002). Expression of CLINK in plant cells releases the RBR-mediated repression of progression from G1 to S-phase, which is detectable by increased expression of cell cycle marker genes and enhanced replication activities (Aronson et al., 2000; Aronson et al., 2002; Lageix et al., 2007). Consequently, CLINK has been suggested to function as a facilitator that augments the viruses' DNA replication, as the pathogen itself does not encode for any replication machinery, but instead utilizes the plant proteins.

The silencing suppressor protein P0 is another example of a virally encoded F-box like protein (Pazhouhandeh et al., 2006; Bortolamiol et al., 2007). It can be found in Poleroviruses that contain a small plus sense RNA genome (Pazhouhandeh et al., 2006). Its F-box motif permits assembly with ASK1 and ASK2 (Pazhouhandeh et al., 2006), and evidence suggests that it targets the protein ARGONAUTE1 (AGO1; At1g48410) for degradation through a SCF^{P0} complex (Bortolamiol et al., 2007). AGO1 is part of the post-transcriptional gene silencing machinery in plants, required as a central host defense mechanism against viral attacks (Mallory and Vaucheret, 2010). Hence, expression of P0 is a likely a counter mechanism by the virus to facilitate its infection and spreading within plant tissues (Pazhouhandeh et al., 2006; Bortolamiol et al., 2007).

The last example stems from research on *Agrobacterium tumefaciens*, a soil-born pathogen that transfers T-DNA and a set of VIRULENCE (Vir) proteins into plant cells (Gelvin, 2006). Once transfected, a T-complex is formed in the plant cell that comprises the T-DNA and the proteins VirD2 and VirE2 (Lacroix et al., 2006). VirD2 sits at the 5'-end of the T-DNA, while VirE2 forms a coat around the DNA strand (Lacroix et al., 2006). To gain access to the nucleus, the T-complex recruits the host-encoded transcription factor VirE2 INTERACTING PROTEIN 1 (VIP1; At1g43700) for importin-based translocation of the T-complex into the nucleus, as well as its subsequent association with chromatin (Tzfira et al., 2001; Li et al., 2005; Lacroix et al., 2008). Agrobacteria also encode for VirF, an F-box protein that is transfected into the plant cells as well, and which can interact with at least three different ASK proteins, ASK1, ASK2, (At5g08590), and ASK10 (At3g21860) (Schrammeijer et al., 2001). In concert with the host-encoded F-box protein VIP1-BINDING F-BOX (VBF; At1g56250), it mediates degradation of VIP1 and VirE2, likely by unpacking the T-DNA in preparation for its chromosomal integration (Schrammeijer et al., 2001; Tzfira et al., 2004; Anand et al., 2012; Zaltsman et al., 2013). Intriguingly, VBF and VIP1 play roles in abiotic and biotic stress responses, respectively, and at least for VBF, expression is induced by Agrobacteria (Zaltsman et al., 2010; Lacroix and Citovsky, 2013), indicating that the bacteria compromises the host's defense pathway to facilitate integration of the T-DNA into the plant genome.

The existence of F-box proteins in viruses and bacteria is even more curious if one considers that the ubiquitin proteasome pathway is classically present only in eukaryotes. Thus, these motifs may have evolved independently from plants or were introduced by horizontal gene transfer. Although at this point only a few examples have been published where pathogens use F-box proteins to facilitate infection of tissues and cells in plants. Further analysis of pathogen genomes, as well as their infection strategies, will likely lead to the identification of additional novel F-box proteins that play critical roles in these processes.

CUL3-BASED E3 LIGASES

Complex composition and structural organization of the different subunits

CUL3-based ligases represent a second large class of CRLs (Figueroa et al., 2005; Gingerich et al., 2005; Weber et al., 2005). Arabidopsis encodes for two CUL3s, CUL3a and CUL3b, which are 88% identical to each other on the amino acid level (Weber et al., 2005). Like CUL1, they bind RBX1 through their C-terminal region, and use their N-terminal domain to mediate assembly with substrate adaptor proteins (Figueroa et al., 2005; Gingerich et al., 2005; Weber et al., 2005; Yoshida et al., 2005). Crystallization of a human CRL3 E3 ligase complex revealed a similar structural organization as described for SCF complexes (Zhuang et al., 2009).

In contrast to SCF complexes, which employ two proteins, an ASK and an F-box protein, to bring substrates to the cullin-RBX1 core, CRL3 E3 ligases only use a single protein for this task. Like ASKs these substrate receptors also contain a BTB/POZ fold that is required to assemble with CUL3 (Dieterle et al., 2005; Figueroa et al., 2005; Gingerich et al., 2005; Weber et al., 2005; Zhuang et al., 2009). Despite related folds, ASK and BTB/POZ proteins interact specifically with either CUL1 or CUL3, respectively. Crystal structure analysis of animal BTB/POZ proteins showed that the domain consists of six α-helices and three β-sheets, forming tightly interwound butterfly-shaped dimers with an extensive hydrophobic interface (Zollman et al., 1994; Ahmad et al., 1998).

BTB/POZ proteins are not as numerous as the F-box family, but they comprise a significant number in Arabidopsis, with 80

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annotated members that are split into ten subfamilies (Gingerich et al., 2005). Besides their BTB/POZ fold, they all contain secondary domains, with the most abundant one being a NPH3 (NON-PHOTOTROPHIC HYPOCOTYL3) fold that can be found in 23 members (Gingerich et al., 2005). For most of these BTB/POZ proteins, however, it is open as to whether or not they indeed function as substrate adaptor subunits of CRL3 E3 ligases, and what biological role they play in plants. An overview of processes linked to the CRL3 complex is shown in Figure 3, and a summary of the substrate adaptors is presented in Table 2.

CRL3 function in higher plants

CUL3a and *CUL3b* exhibit widely overlapping expression patterns and can be found throughout the plant (Figueroa et al., 2005; Weber et al., 2005). They appear to be functionally redundant, since loss of a single member has only mild impacts on development. For example, reports on *cul3a* null mutants have shown slightly delayed flowering time, and a mild far-red light insensitivity (Dieterle et al., 2005), while loss of both CUL3s results in an embryo lethal phenotype with embryogenesis being aborted between the globular to the transient stage (Figueroa et al., 2005). In addition, *cul3*^{hyp} (*cul3*^{hypomorph}) double mutants that lack CUL3b but retain a partially functional CUL3a show ethylene related defects, which correlate with increased amounts of 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE (ACS5; At5g65800), a key enzyme in ethylene biosynthesis (Thomann et al., 2009). *cul3*^{hyp} mutants are also affected in cotyledon development, most obvious by increased numbers, fusion, and aberrant vascular tissue formation. *cul3*^{hyp} mutants are characterized by an overall reduction in root and shoot growth, and further delays in flowering time compared to the *cul3a-1* single mutant (Dieterle et al., 2005; Thomann et al., 2009). These findings emphasized a broader role of CRL3$^{BTB/POZ}$ E3 ligases in plant development, and to dissect their individual roles, the most fruitful approach is again to understand the role of individual substrate receptors.

Phytohormone regulation and pathogen response

The earliest connection between CRL3 E3 ligases and phytohormone signaling comes from work on the ethylene biosynthesis mutant *ethylene overproducer 1 (eto1; At3g51770)* (Wang et al., 2004a). As the name indicates, *eto1* mutants contain significantly elevated levels of ethylene, which result in a constitutive triple

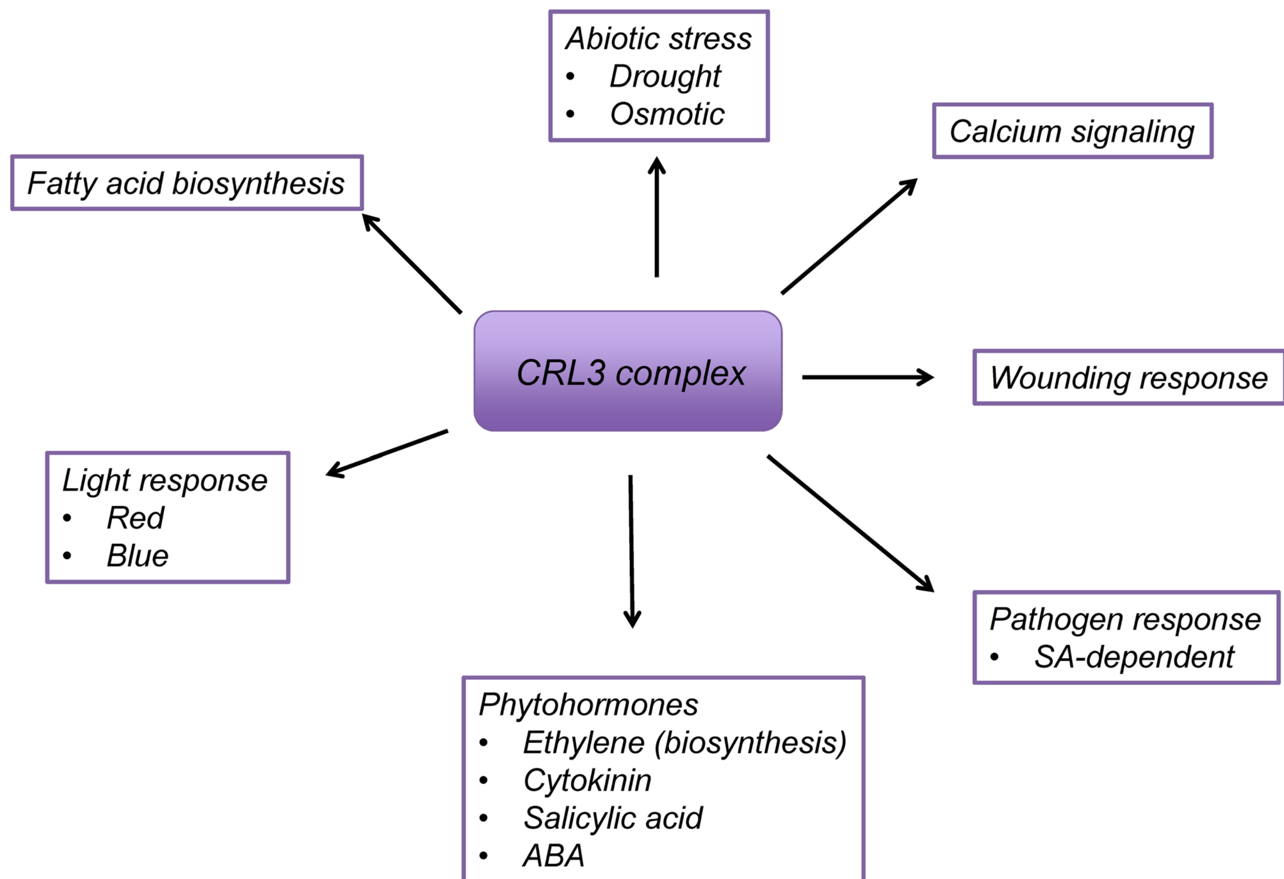

Figure 3. CRL3 complexes and their roles in plant processes.

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Table 2. CRL3 substrate adapters described in this chapter

Protein Name	AGI Name	Proposed Function	References
ARIA	At5g19330	ABA signaling,	Kim et al 2004; Lee et al 2009b
BPM		ABA signaling, ABA-stomatal response,	Lechner et al 2011; Weber et al 2005
-BPM1	At1g19000		
-BPM2	At3g06190	Drought response, wounding response, senescence, ethylene signaling, cytokinin signaling, fatty acid biosynthesis, seed development	Weber & Hellmann 2005; Chen et al 2013
-BPM3	At2g39760		
-BPM4	At3g03740		
-BPM5	At3g21020		
-BPM6	At3g43700		
EOL1 EOL2	At4g02680 At5g58550	Ethylene biosynthesis	Wang et al 2004a; Christians et al 2009
ETO1	At3g51770	Ethylene biosynthesis	Wang et al 2004a; Christians et al 2009
NPH3	At5g64330	Blue light-dependent bending, blue light regulation,	Pedmale & Liscum 2007; Roberts et al 2007
NPR1	At1g64280	SA-dependent pathogen response; cellular redox potential; effector triggered immunity	Spoel et al 2009;
NPR3	At5g45110	SA-dependent pathogen response; effector triggered immunity	Fu et al 2012; Zhang et al 2006
NPR4	At4g19660	Basal pathogen immunity	Fu et al 2012; Zhang et al 2006
SR1IP1	At5g67385	Calcium signaling, pathogen response, enhanced by SA	Zhang et al 2014

response (Roman et al., 1995). Cloning and molecular characterization of *ETO1* revealed that the mutant is affected in a BTB/POZ- TETRATRICOPEPTIDE REPEAT (TPR) protein (Wang et al., 2004a), and that it targets type-II ACS ethylene biosynthesis enzymes for proteasomal degradation (Wang et al., 2004a; Yoshida et al., 2005; Christians et al., 2009). Arabidopsis encodes for two ETO1-like proteins called ETO1-LIKE1 (EOL1; At4g02680) and ETO2-LIKE1 (EOL2; At5g58550), and all three of them participate in controlling ethylene biosynthesis (Wang et al., 2004a; Christians et al., 2009). Of note is that two other ethylene overproducing mutants, *eto2* and *eto3*, are affected in the type II proteins ACS5 and ACS9 (At3g49700), respectively (Chae et al., 2003). Since the mutated ACS5^{eto2} shows increased stability (Chae et al., 2003), it is likely that the corresponding point mutations disrupt its ability to assemble with members of the ETO1/EOL-family.

Perhaps one of the most exiting findings about CRL3 E3 ligases in recent years was the identification that BTB/POZ proteins of the NON-EXPRESSOR OF PATHOGENESIS-RELATED (NPR) family function as receptors of salicylic acid (SA) in SA-dependent defense signaling pathways, with NPR1 (At1g64280) being a long recognized key mediator in this pathway. (Fu et al., 2012; Shi et al., 2012; Pajerowska-Mukhtar et al., 2013). The family comprises four members that have an ankyrin domain in addition to their BTB/POZ fold, and recent work has indicated that they do not all function exclusively as substrate adaptors. In the absence of SA, NPR1 is present as an inactive oligomeric complex. In the presence of SA, together with changes in the cellular redox potential, NPR1 switches to monomeric and dimeric forms that accumulate in the nucleus and bind to TGACG SEQUENCE-SPECIFIC BINDING PROTEIN (TGA) transcription factors, which are involved in controlling expression of defense response genes (Mou et al., 2003; Boyle et al., 2009). For TGA2 (At5g06950) it has been demonstrated that binding is mediated by the BTB/POZ domain of NPR1, and SA-induces a DNA-TGA2-NPR1 ternary complex to promote *PATHOGENESIS-RELATED-1 (PR-1; At2g14610*) gene expression (Rochon et al., 2006; Boyle et al., 2009). Subsequently, NPR1 is phosphorylated in the nucleus, likely while being associated with a TGA transcription factor, and this phosphorylation results in its CUL3-dependent degradation (Spoel et al., 2009). This degradation of phosphorylated NPR1 under SA-inducing conditions is hypothesized to maintain high levels of transcriptional activities by repetitively allowing the formation of new transcription factor complexes with ‘fresh’ NPR1 translocated from the cytosol into the nucleus (Spoel et al. 2009). In addition, it was also recently shown that NPR1 degradation requires the activities of two other NPR members, NPR3 (At5g45110) and NPR4 (At4g19660), that function as negative

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regulators of a SA-response (Zhang et al., 2006; Fu et al., 2012). Biochemical studies demonstrated that NPR3 and NPR4 bind SA with low and high affinities, respectively (Fu et al., 2012; Moreau et al., 2012). Surprisingly, SA promoted NPR1-NPR3 interaction, but disrupted assembly between NPR1 and NPR4 (Fu et al., 2012). This led to currently discussed models in which NPR4 mediates degradation of NPR1 in the absence of SA. Increasing levels of SA in response to pathogens would negatively affect the affinity of NPR4 for NPR1, and allow the cell to build up a basal defense response. It would also provide the opportunity to induce effector-triggered immunity in neighboring cells. In contrast, cells directly attacked by a pathogen may experience very high levels of SA that would promote CRL3^{NPR3}-mediated NPR1 degradation, and induce programmed cell death rather than a cellular defense response.

NPR1 was also recently established to bind SA as well as a metal ion through two cysteine residues (Cys$^{521/529}$) in its C-terminal region (Rochon et al., 2006; Boyle et al., 2009; Wu et al., 2012b). Binding of these two molecules by NPR1 is a requirement to allow formation of a ternary TGA-NPR1-DNA complex and to induce gene expression (Rochon et al., 2006; Wu et al., 2012b). SA/metal ion binding of NPR1 to activate the BTB/POZ protein is discussed together with changes in the cellular redox potential, and NPR3 and NPR4 activities, as an additional regulatory step to fine-tune the SA- and NPR-dependent signal transduction pathway.

Calcium signaling also plays critical roles in the defense response to pathogens. One example is SIGNAL-RESPONSIVE GENE1/CALMODULIN BINDING TRANSCRIPTION ACTIVATOR3 (AtSR1/CAMTA3; At2g22300), which negatively regulates expression of *ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1; At3g48090*), a key protein for both basal resistance as well as effector-triggered immunity in a calcium/calmodulin dependent manner (Du et al., 2009; Garcia et al., 2010). In the search for regulatory steps that control AtSR1 activity, AtSR1 INTERACTING PROTEIN1 (AtSR1IP1) was found. This protein encodes for a BTB/POZ protein with a C-terminal NPH3 domain, and acts as a positive regulator in defense responses with *atsr1ip1* mutants having increased susceptibility to bacterial pathogens (Zhang et al., 2014). Current findings point out that AtSR1IP1 targets AtSR1 for degradation as part of a CRL3^{AtSR1IP1} E3 ligase, and that this process is accelerated by SA-treatment (Zhang et al., 2014). However, it remains open how SA specifically triggers this assembly and promotes AtSR1's degradation through the 26S proteasome pathway.

CRL3 E3 ligases are also involved in ABA signaling by employing BTB/POZ-MATH (BPM) proteins. Arabidopsis encodes for six BPM members that contain a BTB/POZ domain in their C-terminal region and a MATH (Meprin And Traf Homology) fold in their N-terminal part (Weber et al., 2005; Lechner et al., 2011). Recent work has shown binding of their MATH domain with the class I HOMEOBOXLEUCINE ZIPPER (HD-ZIP) transcription factor, HOMEOBOX PROTEIN 6 (ATHB6; At2g22430) (Lechner et al., 2011). ATHB6 is known as a negative response regulator within the ABA signal transduction pathway (Himmelbach et al., 2002), and current evidence strongly suggests that BPM-ATHB6 assembly destabilizes ATHB6 (Lechner et al., 2011). Therefore, the corresponding CUL3^{BPM} E3 ligase can likely be considered a positive ABA response mediator. This is further supported by the phenotypes seen in the transgenic lines with reduced *BPM* expression levels, which show reduced responsiveness in stomata closure after treatment with ABA (Lechner et al., 2011). The BPMs also interact with several ETHYLENE RESPONSE FACTOR/APETALA2 (ERF/AP2) transcription factors, a family of proteins that are associated with phytohormone signal transduction, abiotic stress response, and metabolism (Gutterson and Reuber, 2004; Stepanova and Alonso, 2005; Baud et al., 2009; Xu et al., 2011). Interaction of BPMs with ERF/AP2s has been described for RELATED TO APETALA2.4/WOUND INDUCED DEDIFFERENTIATION 1 (RAP2.4/WIND1; At1g78080) and WRINKLED1 (WRI1; At3g54320) (Weber and Hellmann, 2009; Chen et al., 2013). RAP2.4 is involved in drought and wound stress responses, and senescence control, as well as ethylene and cytokinin-related developmental processes (Lin et al., 2008; Xu et al., 2010a; Iwase et al., 2011), while WRI1 is a key regulator of fatty acid biosynthesis in seeds and appears to have roles in early seedling development (Cernac et al., 2006; To et al., 2012; Ma et al., 2013). Current studies indicate that CRL3^{BPM} complexes interact directly at the DNA level with their ERF/AP2 substrates, and this assembly results in their proteolytic degradation (Chen et al., 2013). It is likely that this also holds true for CRL3^{BPM}-ATHB6 interactions.

Intriguingly, ERF/AP2 proteins are not only targeted by CRL3 E3s, but they are also recognized by other E3 ligases (Dong et al., 2006; Catala et al., 2007; Weber and Hellmann, 2009; Cheng et al., 2012; Chen et al., 2013), indicating that E3 ligase-dependent regulation of ERF/AP2 proteins is a broadly utilized scheme of controlling their activities in plants.

Finally, it is relevant to mention that the BTB/POZ protein ARM REPEAT PROTEIN INTERACTING WITH ABF2 (ARIA; At5g19330) also functions as a positive ABA response mediator (Kim et al., 2004; Lee et al., 2009b). Although the protein could serve like BPMs as a substrate adaptor to CRL3s, no data are currently available that support such a role in plants. ARIA interacts with two other positive ABA-response modulators, the leucine zipper transcription factor ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 2 (ABF2; At1g45249) and the ERF/AP2 protein ARIA-INTERACTING DOUBLE AP2 DOMAIN PROTEIN (ADAP; At1g16060) (Kim et al., 2004; Lee et al., 2009b). However, since all of these proteins are positive mediators of an ABA response, it appears to be unlikely that ARIA would mediate instability of ABF2 and ADAP. Rather their assemblies may be of a CRL3-independent nature, but required to efficiently promote transcription of ABA responsive genes.

CRL3 in light regulation

CRL3 E3 ligases, like SCF E3s, participate in red and blue light signal transduction pathways. So far the data on red light responses are limited to *cul3* mutants displaying a decreased far-red sensitivity. However, major progress has been made in understanding the role of CRL3s in mediating blue light (BL) response (Pedmale and Liscum, 2007; Roberts et al., 2011). A substrate adaptor that functions in this pathway is NON-PHOTOTROPIC HYPOCOTYL3 (NPH3; At5g64330), a protein with an N-terminally located BTB/POZ domain, a centrally located NPH3 fold, and a

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coiled-coil domain in its C-terminal region (Stogios et al., 2005). *nph3* was originally identified in a screen for mutants that showed reduced BL dependent bending of hypocotyls, a process that is initiated by PHOTOTROPIN (PHOT; At3g45780) photoreceptors (Christie, 2007). *cul3*^{hyp} double mutants also show a reduced BL dependent curvature response of the hypocotyl, and biochemical studies demonstrated that a CRL3^{NPH3}-PHOT1 complex is formed upon BL exposure (Roberts et al., 2011). Under low intensity BL this assembly leads to a mono/multi-ubiquitylation of PHOT1, which does not destabilize the protein. Under high intensity BL, PHOT1 is also poly-ubiquitylated and degraded (Roberts et al., 2011). These different ubiquitylation patterns are suggested to function as regulatory steps to control PHOT1 activities: low BL may induce translocation, while high BL promotes degradation of PHOT1, to impact the overall BL sensitivity of the cell (Roberts et al., 2011). These findings are a good example that E3 ligases do not necessarily always cause degradation of their substrates, but that UBQ conjugation may function as a regulatory tool to modulate protein activity, similar to a phosphorylation event.

CUL4-BASED E3 LIGASES

Complex composition and structural organization of different subunits

The most recently described class of cullin-based E3 ligases are CRL4 complexes, which can be found throughout all plants and across many eukaryotes characterized so far (Biedermann and Hellmann, 2011). Arabidopsis CUL4 shows greater similarity to human Cul4 (63.5%) than to its closest plant homolog CUL3b (59.1%), emphasizing the conserved nature of this E3 ligase among different organisms (Bernhardt et al., 2006). As in SCF and CRL3 E3 ligases, CUL4 uses its C-terminal region to assemble with RBX1, while the N-terminus serves to bind DNA DAMAGED BINDING PROTEIN 1 (DDB1), a 125 kDa protein that facilitates binding of substrate adaptors to the core E3 ligase (Angers et al., 2006; Bernhardt et al., 2006; Lee et al., 2008). Crystallization of human Ddb1 showed that the protein consists of three β-propellers, BPA, BPB, and BPC, which form a clam-like structure (Angers et al., 2006). While the BPB domain is required for docking to the cullin, BPA and BPC serve as binding sites for the substrate adaptors (Angers et al., 2006; Scrima et al., 2008).

Two homologs of Ddb1, DDB1a (At4g05420) and DDB1b(At4g21100), can be found in the Arabidopsis genome that are 91% identical to each other with highly overlapping expression patterns in the plant (Bernhardt et al., 2006; Bernhardt et al., 2010). DDB1 proteins are able to interact with a wide variety of proteins collectively known as DDB1 CUL4 ASSOCIATED FACTORS (DCAF) (Biedermann and Hellmann, 2011). DCAF proteins are characterized by the presence of multiple WD40 repeats, often with at least one of these repeats ending in an arginine (WDxR). Additionally, a conserved 16-17 amino acid sequence called the DDB1 BINDING WD40 (DWD)-box is also able to facilitate interaction of substrate adaptors with DDB1 (Lee et al., 2008). In many cases the importance of the last arginine residue is seen through abolishment of interactions with DDB1 upon mutation (Chen et al., 2010; Castells et al., 2011; Pazhouhandeh et al., 2011). 119 proteins with a WDxR motif are encoded in the Arabidopsis genome, and 85 of these contain at least one DWD box leading to a wide diversity of proteins that can serve as potential substrate adaptors for a CRL4 core complex (Lee et al., 2008). Similar numbers have also been reported for rice (Lee et al., 2008), indicating that most plants encode a high number of DCAF proteins. However, at this point only a fraction of these have actually been tested for assembly with DDB1 leaving it open to what extent individual DCAF proteins indeed play a role as CRL4 E3 ligase substrate adaptors.

CRL4 function in higher plants

Arabidopsis *CUL4* is expressed throughout the entire plant, and given the potentially high number of CRL4 substrate adaptors, it is not surprising that phenotypic analysis of *cul4* mutants revealed defects in a wide range of plant-related processes (Bernhardt et al., 2006; Chen et al., 2006). Although null mutants have not been described so far, reduced levels of CUL4 already result in irregular cotyledon and leaf development, vascular vein orientation, and stomatal development, as well as reduced lateral root growth and dwarfism (Bernhardt et al., 2006; Chen et al., 2006). Additionally, a constitutive photomorphogenesis phenotype is exhibited in *CUL4* co-suppression lines, which also correlates with altered expression of light-regulated genes (Chen et al., 2006).

Likewise, the two DDB1 proteins have been implicated in a wide variety of developmental processes such as leaf and lateral root numbers, the flowering time point, silique size, and longer hypocotyls, but also with photomorphogenesis and ultraviolet (UV)-tolerance (Schroeder et al., 2002; Molinier et al., 2008; Al Khateeb and Schroeder, 2009; Bernhardt et al., 2010). Of note is that *DDB1a* null mutants only show minor phenotypic changes when compared to wild type, while loss of *DDB1b* results in an embryo lethal phenotype (Schroeder et al., 2002; Bernhardt et al., 2010). However genetic analysis of *ddb1a*/*ddb1b* double mutants demonstrated that DDB1a is also involved in embryogenesis, and corresponding mutants arrest between the globular to late heart stage (Bernhardt et al., 2010).

In the following we will focus on specific processes in which CRL4 E3 ligases have been established to function. An overview of processes linked to the CRL4 complex is shown in Figure 4, and a summary of the substrate adaptors discussed below is presented in Table 3.

Phytohormones and abiotic stress response

Several studies have linked CRL4 E3 ligases to phytohormones, especially in ABA-related processes. Recently, a family of DWD proteins called DWD HYPERSENSITIVE TO ABA (DWA) family that have been shown to affect ABA signaling, are able act like DCAF proteins and interact with the CRL4 complex (Lee et al., 2010; Lee et al., 2011). Two of the proteins in this family, DWA1 (At2g19430) and DWA2 (At1g76260), are able to interact with each other, as well as with DDB1b, suggesting that they may work in concert with each other to bring substrates to the CRL4 complex (Lee et al., 2010). Root growth assays showed

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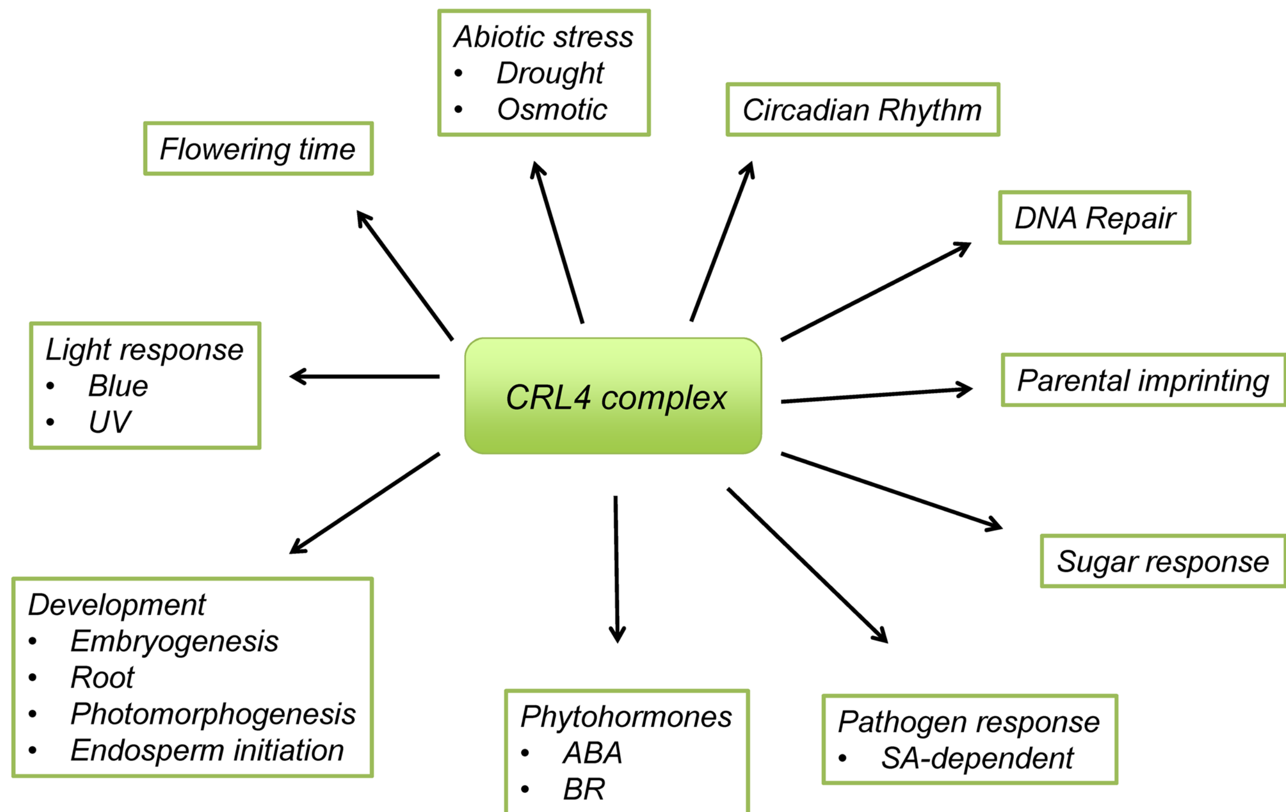

Figure 4. CRL4 complexes play a wide role in plant processes.

that the corresponding *dwa* null mutants develop shorter primary roots in the presence of ABA and NaCl, and this was even more pronounced in a *dwa1*/*dwa2* double mutant (Lee et al., 2010). The hypersensitivity characterized the two proteins as negative regulators in ABA signaling. In search for potential CRL4$^{DWA1/DWA2}$ substrates, ABA-INSENSITIVE5 (ABI5; At2g36270), a leucine zipper transcription factor, was identified. The protein physically assembles into a complex with DWA1 and DWA2, as well as with CUL4 (Lee et al., 2010). In addition, ABI5 is unstable in a 26S proteasome dependent manner, but becomes stable in a *cul4* or *dwa* mutant background (Lee et al., 2010). A third member of the DWA family, DWA3 (At1g61210), is also able to interact with DDB1b and CUL4 (Lee et al., 2011). Although it was suggested to function as a negative ABA response regulator as well, the protein does not assemble with the other two DWA proteins or with ABI5 (Lee et al., 2011), leaving it unclear how DWA3 specifically acts within the pathway.

ABA-HYPERSENSITIVE DCAF1 (ABD1; AT4G38480), another DCAF protein, can also interact with DDB1 proteins, and is unstable in a CRL4-dependent manner (Seo et al., 2014). Like DWA proteins, ABD1 is considered to be a negative ABA-response regulator since its loss results in ABA and NaCl hypersensitivity. Interestingly, while ABD1 can also assemble with ABI5, no interaction with the previously discussed DWA family of proteins is observed (Seo et al., 2014). This suggests that ABD1 works independent from the DWA proteins and that the regulation of ABI5 occurs through a coordination of multiple E3 ligase complexes (Seo et al., 2014).

PLEIOTROPIC REGULATORY LOCUS1 (PRL1; AT4G15900) is another example of a potential CRL4 substrate adaptor that is able to interact with DDB1 (Lee et al., 2008). *prl1* mutants show ABA, cytokinin, and sugar hypersensitivity apparent by reduced root elongation growth when treated with either phytohormone or glucose, respectively (Lee et al 2008). In conjunction, a wide variety of ABA-, cytokinin-, and sugar responsive genes are up-regulated in *prl1* mutants when compared to wild type plants (Lee et al., 2008). PRL1 is able to interact with ARABIDOPSIS SUCROSE NON-FERMENTING-1 KINASE HOMOLOG 10 (AKIN10; At3g01090) and AKIN11 (At3g29160) that are involved in metabolic signaling and development (Fragoso et al., 2009; Tsai and Gazzarrini, 2012). Previous work showed that AKIN10 and AKIN11 are able to interact with ASK1, but this interaction is disrupted by PRL1 leading to the hypothesis that PRL1 and ASK1 are competing for AKIN10 and AKIN11 binding (Farras et al., 2001). Alternatively, it was suggested that PRL1 works together with CUL4 in order to target either AKIN10 and/or AKIN11 for degradation via the 26s proteasome. This hypothesis is supported by *in vitro* assays using whole plant protein extract,

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Table 3. CRL4 substrate adaptors described in this chapter

Protein Name	AGI Name	Proposed Function	References
ABD1	AT4G38480	ABA signaling, salt tolerance	Seo et al 2014
ATCSA	AT1G27840	NER, UV-sensitivity	Biedermann & Hellmann 2010; Zhang et al 2010
COP1	AT2G32950	Photomorphogenesis, flowering time, UV-induced morphogenesis	Chen et al 2010; Huang et al 2013b; Jang et al 2008
COP10	AT3G13550	Photomorphogenesis, ubiquitin ligase activity	Lau & Deng 2009;
DDB2	AT5G58760	NER, UV- sensitivity	Biedermann & Hellmann 2010; Molinier et al 2008
DET1	AT4G10180	Photomorphogenesis, circadian rhythm, DNA repair	Schroeder et al 2002; Bernhard et al 2006
DWA1 DWA2	AT2G19430 AT1G76260	ABA signaling, salt tolerance	Lee et al 2010
DWA3	AT1G61210	ABA signaling	Lee et al 2011
FY	At5g13480	Flowering, autonomous promotion pathway	Lee et al 2008
MSI1	AT5G58230	Parental imprinting, endosperm initiation	Kohler et al 2003; Dumbliauskas et al 2011
MSI4	AT2G19520	Flowering, autonomous promotion pathway, epigenetics	Pazhouhandeh et al 2011;
PRL1	AT4G15900	ABA signaling, cytokinin signaling, sugar sensitivity	Lee et al 2008; Fragoso et al 2009
SPA -SPA1 -SPA2 -SPA3 -SPA4	 At2g46340 At4g11110 At3g15354 At1g53090	Photomorphogenesis, flowering time, UV-induced morphogenesis,	Chen et al 2010; Huang et al 2013b; Zhu et al 2008
TRIP1/eIF3i	AT2G46280	Brassinosteroid signaling	Jiang & Clouse 2001; Lee et al 2008

in which AKIN10 degradation is reduced in *prl1* and *cul4* mutant extracts when compared to wild type extracts (Lee et al., 2008). However, the exact interplay between SCF and CRL4 E3 ligases on AKIN10 and AKIN11 remains unclear.

A last example for DWD proteins and CRL4 E3 ligases involved in phytohormone signal transduction comes from TGF-β RECEPTOR INTERACTING PROTEIN 1/EUKARYOTIC TRANSLATION INITIATION FACTOR 3i (TRIP-1/eIF3i; At2g46280), which is believed to participate in translation initiation as part of the larger eIF3 complex (Jiang and Clouse, 2001; Lee et al., 2008). TRIP-1 physically assembles with and is phosphorylated by the serine/threonine receptor kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1; At4g39400), a major component of the brassinosteroid signaling pathway (Ehsan et al., 2005). Although its role in brassinosteroid signaling is open, TRIP-1 co-immunoprecipitates with DDB1b and CUL4, and so is very likely to play a role in phytohormone signaling pathway as part of a CRL4^{TRIP-1} E3 ligase complex (Lee et al., 2008).

Photomorphogenesis and flowering control by CRL4

CONSTITUTIVELY PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA (COP/DET/FUS) is a group of proteins identified through genetic screening to play a major role in the transition from etiolated to de-etiolated development and many of these proteins have been shown to interact to form distinct CRL4 complexes (Hardtke and Deng, 2000).

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DET1 (At4g10180) and COP10 (At3g13550) have been demonstrated to form a complex with DDB1 and CUL4 (Yanagawa et al., 2004; Bernhardt et al., 2006). DET1 has been primarily brought into context with repression of photomorphogenesis, but also with circadian rhythms and, as discussed, with DNA repair (Schroeder et al., 2002; Yanagawa et al., 2004; Song and Carre, 2005; Castells et al., 2010; Castells et al., 2011). COP10 also functions as a negative regulator of photomorphogenesis, and encodes for an E2-like protein without catalytic activity that is proposed to enhance the conjugating activities of other E2s (Lau and Deng, 2009). Of note is that both proteins do not contain a DDB1-interacting motif, and it remains open how they assemble with DDB1. It is also currently unclear exactly how a corresponding $CRL4^{DET1/COP10}$ E3 ligase functions in context of repressing photomorphogenesis.

The COP10-DET1-DDB1 (CDD) complex, however, can serve as an adaptor module that facilitates binding of additional substrate receptors (Lau and Deng, 2012). Recent work supports this notion since the complex recruits an additional protein called DET1-, DDB1-ASSOCIATED1 (DDA1) (Irigoyen et al., 2014). The protein does not contain a DWD motif or any other obvious domain that is known to mediate interaction with DDB1. However, it can assemble in yeast-2-hybrid assays with the BPA domain of DDB1, and presumably contains another, yet unidentified domain that can mediate this interaction (Irigoyen et al., 2014). DDA1 also interacts with members of the PYRABACTIN RESISTANCE/PRL-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS (PYR/PYL/RCAR) family of ABA receptors, and its overexpression leads to increased instability of PYL8 (At5g53160), which correlates with reduced ABA sensitivity (Irigoyen et al., 2014). These data overall support the idea that DDA1 is the actual substrate receptor to the $CRL4^{CDD}$ E3 ligase, and it also opens up the possibility that additional, unknown substrate adaptor subunits assemble into the $CRL4^{CDD}$ complex.

COP1 (At2g32950) is a master regulator of photomorphogenesis (Osterlund et al., 1999). Under dark conditions, it acts as an E3 ligase that in concert with SUPPRESSOR of PHYTOCHROME A (SPA) proteins is able to target various transcription factors that function as positive key regulators of photomorphogenesis, such as ELONGATED HYPOCOTYL5 (HY5; At5g11260), LONG AFTER FAR-RED LIGHT 1 (LAF1; At4g25560), or LONG HYPOCOTYL IN FAR-RED1 (HFR1; At1g02340), for proteasomal degradation (Jang et al., 2005; Jang et al., 2007). SPA proteins form a small family with four members in Arabidopsis that form homo- and heterodimers with each other, and which can assemble with dimerized COP1 (Zhu et al., 2008). Both COP1 and SPAs are DWD proteins, and can further assemble with CUL4 and DDB1 in the plant (Chen et al., 2010).

It is not fully clear what the functional advantage is of forming a CRL4-COP1-SPA complex. A possible explanation could be that it positively modulates certain activities of COP1. Evidence for this possibility comes from COP1's role in flowering time control (Jang et al., 2008; Zuo et al., 2011). COP1 also participates in controlling stability of CO, which as previously discussed can induce expression of *FT* a critical transcription factor for inducing the switch from a vegetative to a reproductive apical meristem (Thomas, 2006). Of note is that *cop1* and *cul4* mutants show elevated levels of *FT* expression, early flowering phenotypes, and photomorphogenesis under darkness conditions, all of which are enhanced in corresponding double mutants (Chen et al., 2010).

COP1 also plays a role in photomorphogenesis under low-fluence and long-wavelength UV-B-induced environments (Favory et al., 2009). Under dark settings it acts as a repressor of photomorphogenesis by promoting degradation of key transcription factors. In contrast, under UV-conditions it positively modulates this developmental step by stabilizing HY5. This change in function is induced by the UV-receptor UV RESISTANCE LOCUS8 (UVR8; At5g63860), which assembles with COP1 after UV-exposure, and induces a dissociation of COP1-SPA from the CRL4-core complex (Wu et al., 2012a; Huang et al., 2013b; Huang et al., 2014). Thus different environmental light situations appear to control what kind of interactions COP1 may undergo and thereby define specific activities of the E3 ligase.

CRL4s have also been implicated with flowering time control independently of COP1 by regulating levels of FLC (FLOWERING LOCUST C; At5g10140), a central negative regulator of flowering (Feng et al., 2011). One such connection comes from FY (FLOWERING LOCUS Y; At5g13480), a DWD protein that is part of the autonomous flowering pathway, and which represses FLC activities. FY has been shown to interact with DDB1b and to co-immunoprecipitate with CUL4, providing strong evidence that FLC control runs through $CRL4^{FY}$ activities (Lee et al., 2008). Furthermore, a CRL4 E3 ligase is also involved in the epigenetic control of *FLC* expression by assembling with the DWD protein MULTI-COPY SUPPRESSOR OF IRA4 (MSI4; At2g19520) and the CLF-POLYCOMB REPRESSIVE COMPLEX 2 (PRC2)-like complex (Pazhouhandeh et al., 2011). MSI4, like FY, is known as a key regulator within the autonomous pathway (Marquardt et al., 2006), while PRC2-like complexes can mediate trimethylation of histone 3 (H3K27), a negative regulatory step to down-regulate gene expression (Guitton and Berger, 2005). A target of PRC2-like complexes in Arabidopsis is the *FLC* promoter, and current work indicates that $CRL4^{MSI4}$ functions as a positive regulator of PRC2 activities (Pazhouhandeh et al., 2011). Thus, it is likely that $CRL4^{MSI4}$ modulates PRC2 by monoubiquitylation of PRC2 subunits, rather than causing their proteasomal degradation by polyubiquitylation (Pazhouhandeh et al., 2011). Of note is that DDB1 also interacts with other members of the MULTI-COPY SUPPRESSOR OF IRA (MSI; At5g58230) family (Bernhardt et al., 2010; Dumbliauskas et al., 2011), and that the assembly with MSI1 and PRC2-like complexes controls parental imprinting and endosperm initiation in seed development (Kohler et al., 2003; Leroy et al., 2007; Dumbliauskas et al., 2011).

CRL4 E3 ligases and DNA repair

Changes in DDB1 or CUL4 levels critically affect UV-sensitivity of plants (Molinier et al., 2008; Al Khateeb and Schroeder, 2009). Regarding the conserved nature of CUL4 and DDB1 this is not surprising since DDB1 was first described in context with UV sensitivity in humans, and the plant DDB1 orthologs are around 52% identical to their human counterparts (Payne and Chu, 1994; Dualan et al., 1995; Bernhardt et al., 2010).

Exposure to UV-B and UV-C light can result in DNA lesions most often represented by either cyclobutane pyrimidine dimers (CPD) or (6-4) pyrimidine-pyrimidine photoproducts (6-4PP). In plants, the predominant repair pathway for these lesions runs

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through the activity of photolyases, which require light to photoreactivate the damaged sites (Yi and He, 2013). Alternatively, such sites can also be repaired by Nucleotide Excision Repair (NER), which is a light-independent DNA repair pathway (Biedermann et al., 2011). Two DWD proteins, DNA DAMAGE BINDING PROTEIN 2 (Ddb2) and COCKAYNE SYNDROME A (CSA), play critical roles in detecting UV-induced DNA lesion, and activating their subsequent repair through the NER (Biedermann et al., 2011).

DDB2 was first identified in human patients that suffer from *xeroderma pigmentosum* (XP) disease. XP causes UV hypersensitivity, and most of the corresponding patients carried mutations in Ddb2 that prevented complex formation with Ddb1 (Wittschieben & Wood 2003). DDB2 is also able to bind UV-induced DNA lesions with high affinity, which serves as a mark for the NER machinery to repair the corresponding sites (Scrima et al., 2008; Biedermann et al., 2011). Complex formation with DDB1 and CUL4 is necessary to degrade DDB2 and to make the damaged sites accessible for the repair machinery (Matsuda et al., 2005; Biedermann et al., 2011; Fischer et al., 2011).

CSA was also first identified in humans, but in contrast to DDB2 it cannot bind directly to DNA. Rather, CSA assembles with RNA polymerases that are stalled at DNA lesions, and this in turn serves as a mark for the repair machinery. Like DDB2, CSA can assemble with DDB1 and this results in degradation of CSA, and also the RNA polymerase, again making the damaged sites accessible for DNA repair (Groisman et al., 2003; Fischer et al., 2011).

Both DDB2 and CSA are functionally conserved between animals and plants, and loss of either protein results in UV hypersensitivity, and reduced DNA repair activities (Molinier et al., 2008; Biedermann and Hellmann, 2010; Zhang et al., 2010). However, hypersensitivity is only observed when plants are kept in the dark after UV-exposure to prevent photolyases from functioning (Biedermann and Hellmann, 2010). In addition, both proteins assemble with CUL4 and DDB1 proteins *in planta*, and increased stability can be observed in *cul4* and *ddb1a* mutants, respectively, demonstrating that plant CSA and DDB2 proteins are actual targets of CRL4 activities (Molinier et al., 2008; Biedermann and Hellmann, 2010; Zhang et al., 2010).

Arabidopsis DDB2 (At5g58760) is located to the nucleus, and is expressed mainly in roots and flowers (Biedermann and Hellmann, 2010). While *DDB2* gene expression is up-regulated upon UV exposure, the protein is unstable in a UV-dependent fashion (Molinier et al., 2008; Biedermann and Hellmann, 2010). Intriguingly, UV-dependent instability of DDB2 is likely regulated by the levels of DDB1 present in the nucleus, since DDB1a is rapidly translocated from the cytosol into the nucleus upon UV exposure (Molinier et al., 2008).

Incidentally, DET1, a protein originally identified in a screen for mutants showing a constitutive photomorphogenesis phenotype (Ang and Deng, 1994), has also been shown to interact with DDB1, and to be involved in UV tolerance and potentially repair (Bernhardt et al., 2006; Castells et al., 2011). Evidence here comes from *det1* knockout mutants displaying UV-hypersensitivity, which is further enhanced in *det1 ddb2* double mutants (Castells et al., 2011). In addition, overexpression of DET1 also results in reduced UV photoproduct removal after UV irradiation, suggesting that cellular DET1 levels are critical for efficient NER activities (Castells et al., 2011). It was further noted that DET1 is degraded in a CRL4- and UV-dependent manner, but that DDB2 is comparably stable in a *det1* mutant background, even after UV irradiation (Castell et al 2011). Overall these findings suggest a cooperative mode of activity between DET1 and DDB2 in context with CRL4 and NER actions.

In Arabidopsis two homologues of the human CSA can be found denoted as *ATCSA-1/CSAat1A (At1g27840)* and *CSAat1B (At1g19750)* (Biedermann and Hellmann, 2010; Zhang et al., 2010). Expression of the two genes is seen in almost all tissues with exceptions present in anthers and seeds, and no UV-dependent induction was detected (Biedermann and Hellmann, 2010; Zhang et al., 2010). ATCSA-1 and CSAat1B are localized in the nucleus, often near areas that have been described as transcriptionally active regions (Biedermann and Hellmann, 2010; Zhang et al., 2010). Loss of either gene causes UV-hypersensitivity, which correlates with reduced DNA repair activities (Biedermann and Hellmann, 2010; Zhang et al., 2010). Interestingly, overexpression of *ATCSA* also results in UV-hypersensitivity as observed for *DET1*, suggesting that appropriate dosages of these proteins are highly critical to coordinate efficient CRL4 activities with the DNA repair process (Biedermann and Hellmann, 2010; Castells et al., 2011).

THE ANAPHASE PROMOTING COMPLEX OR CYCLOSOME

Complex composition and structural organization of the different subunits

The ANAPHASE PROMOTING COMPLEX or CYCLOSOME (APC/C) complex is highly conserved among eukaryotes and represents by far the largest of the four cullin-based E3 ligases known in plants. Plant APC/Cs consist of at least 11 core subunits (Capron et al., 2003a). APC2 (At4g36920) and APC11 (At3g05870) share homology to cullins and RBX1, respectively (Gieffers et al., 2001; Zhang et al., 2013). Accordingly, they function as catalytic subunits in substrate recognition, binding of the E2-UBQ, and UBQ transfer onto the substrate (Zhang et al., 2013). Structural and functional analysis of the human and plant APC/C complexes further shows that the subunits 1, 4, 5, 6, and 8 serve as scaffolding moieties, while APC3 and APC7 (2g39090) participate in binding of APC10 (At2g18290), which together, with APC2, are the two APC core subunits that are involved in substrate binding (Van Leene et al., 2010; Zhang et al., 2013). Except for APC3, for which two related genes can be found in the Arabidopsis genome (APC3a/CELL DIVISION CYCLE27A (CDC27a; At3g16320) and APC3b/HOBBIT/CDC27b (HBT; At2g20000)), all other APC subunits are present as single copies (Blilou et al., 2002; Capron et al., 2003a). A variety of members contain TPR repeats (APC3, APC6, APC7 and APC8) for protein-protein interaction, while other subunits have domains for substrate recognition (DOC HOMOLOGY/IR motif in APC10; At2g18290), or domains of unknown function, such as the PC (Proteasome Cyclosome) repeats in APC1 (At5g05560) (Capron et al., 2003a; Barford, 2011; Zhang et al., 2013). Additional co-activator subunits are required for full activity of the complex. These facilitate binding of specific substrate proteins for targeted ubiquitylation by the APC/C. Two structurally related WD40-repeat protein families characterized as co-activators in plants and animals are CELL

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DIVISION CYCLE20/FIZZY (CDC20/FZ) and CDC20 HOMOLOG1/FIZZY-RELATED (CDH1/FZR). In Arabidopsis they comprise six and three members, respectively (Capron et al., 2003b; Vodermaier et al., 2003; Fulop et al., 2005; da Fonseca et al., 2011), and CDH1/FZR members in plants are also known as CELL CYCLE SWITCH52 (CCS52) proteins. We will designate the co-activators as CDC20 and CCS52, respectively, when the plant proteins are discussed. An overview of processes linked to the APC/C complex is shown in Figure 5, and a summary of the substrate adaptors discussed below is presented in Table 4.

The Cell Cycle Control and APC/C

The major function of the APC/C complex in eukaryotic organisms is cell cycle control (Pesin and Orr-Weaver, 2008). Substrates of the APC/C complex normally contain one of two degron motifs, either a D-box (destruction box) or a KEN box (Glotzer et al., 1991; Pfleger and Kirschner, 2000). The D-box consists of a nine amino acid long highly variable motif (R-X-X-L-X-X-X-X-N) in which mainly the R (arginine) and L (leucine) are conserved (Glotzer et al., 1991). The seven amino acid long KEN box (K-E-N-X-X-X-N) is primarily conserved in its first three amino acid residues K (lysine), E (glutamate), and N (asparagine) (Pfleger and Kirschner, 2000). Proteins carrying a D- or KEN-box are recognized by CDC20/FZ or CDH1/FZR1/CCS52 and can be targeted for proteolytic degradation through a corresponding APC/C complex.

In animals, it is well established that the APC/C complex is required for the separation of sister chromatids through participation of the co-activator Cdc20/FZ, which targets the securin protein and the mitotic cyclins at the metaphase/anaphase transition for 26S proteasome-dependent proteolysis to progress into anaphase. Securin degradation is required to activate separase, a protease that opens up the cohesin complex ring, disrupting the physically attached sister chromatids (Raff et al., 2002; Huang et al., 2005). Progression through these different steps widely depends on the phosphorylation status of participating proteins. For example, the cyclin B-dependent kinase Cdk1 phosphorylates securin close to its D- and KEN-box motifs preventing its recognition by APC/C $^{Cdc20/FZ}$ (Holt et al., 2008). Cdk1 also prevents early assembly of Cdh1/Fzr1 with the APC/C core complex by keeping the substrate receptor in a phosphorylated state (Crasta et al., 2008). Spindle-associated and cytosolic cyclin B are proposed to be degraded by APC/C$^{Cdc20/FZ}$ and APC/C$^{Cdh1/Fzr1}$ complexes, respectively, which in turn inactivates Cdk1 (Raff et al., 2002). In addition, the APC/C substrate adaptor Cdc20/FZ itself is ubiquitylated by APC/C$^{Cdh1/Fzr1}$ in late anaphase and G1, while Cdh1/Fzr1 is also ubiquitylated by the SCF$^{\beta\text{-Trcp}}$ complex in late G1 to S-phase (Fukushima et al., 2013).

In plants, the understanding of the molecular action of progression into anaphase is still poorly understood. Securin homo-

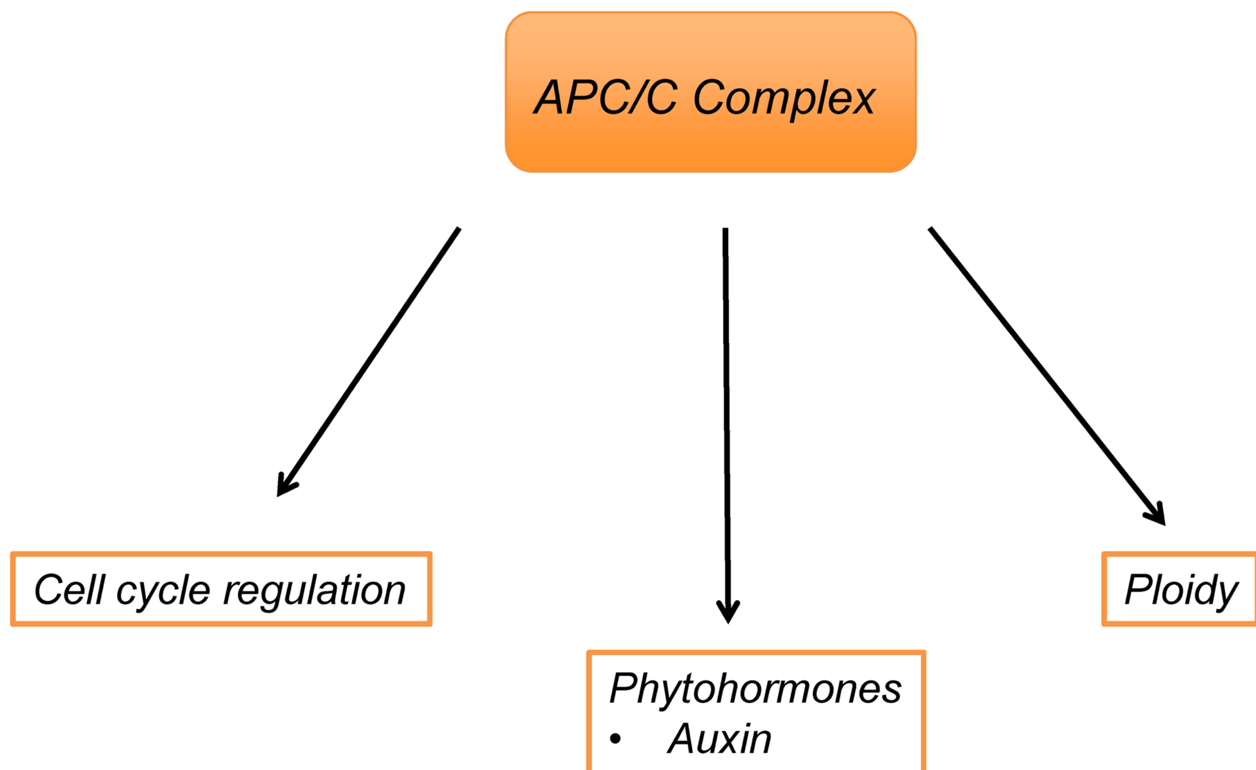

Figure 5. The APC/C complex and its functional role in plants.

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Table 4. APC substrate adapters described in this chapter

Protein Name	AGI Name	Proposed Function	References
APC2	At4g36920	Megagametogenesis	
APC6 APC10	At1g78770 At2g18290	Leaf epidermal development, vascular vein development, shoot development, ploidy	Marrocco et al 2009; Perez-Perez et al 2008
CCS52A1	At4g22910	Ploidy, root meristem (elongation zone), pathogen response	Vanstraelen et al 2009;
CCS52A2	At4g11920	Ploidy, root meristem (quiescent center, stem cells),	Lammens et al 2008
CCS52B	At5g13840	Endocycle control, pathogen response,	Tarayre et al 2004
CDC20.1 CDC20.2	At4g33270 At4g33260	Cell cycle regulation,	Kevei et al 2011
CDC27a	At3g16320	Gametogenesis, embryogenesis	Blilou et al 2002; Perez-Perez et al 2008
HBT	At2g20000	Gametogenesis, embryogenesis, auxin insensitivity, ploidy	Blilou et al 2002; Perez-Perez et al 2008
OsMOC1	Q84MM9	Tillering control	Xu et al 2012
OsRAA1	AAT94064	Spindle apparatus	Xu et al 2010b

logs are not apparent in plant genomes and functionally related proteins having not yet been identified. However, other components controlling the metaphase/anaphase transition are conserved among animals and plants, such as the spindle assembly checkpoint (Caillaud et al., 2009), which is known to negatively regulate APC/C activity in animals. Plants also have a separase-related enzyme that cleaves the cohesin complex ring to promote chromatid separation (Wu et al., 2010). Additionally, arrest in metaphase of tobacco BY2 cells when treated with the 26S proteasome inhibitor MG132 strongly supports that the ubiquitin proteasome pathway is involved in the transition from metaphase to anaphase (Genschik et al., 1998). However analysis of female gametogenesis in *apc2* null mutants showed no arrest in metaphase, although cell cycle control is affected in these plants (Capron et al., 2003b). These findings indicate that the steps regulating the transition to anaphase may be different from that known in animals.

Another problem in analyzing the specific molecular steps in which plant APC/C complexes are active within the mitotic cycle, is that comparably little data has been generated on substrates directly targeted by the complex. Many mitotically expressed plant A- and B-type cyclins are reported to contain D-boxes (Capron et al., 2003a), but current analyses are mainly restricted to a more indirect approach using cyclin:reporter genes. A couple of direct targets have been shown in rice. For example, ORYZA SATIVA ROOT ARCHITECTURE-ASSOCIATED1 (OsRAA1), is a D-box containing protein that associates with the spindle apparatus, and is discussed to function as a negative regulator of the progression from meta- to anaphase (Xu et al., 2010b); and another substrate is MONOCULM1 (MOC1) which is involved in tillering control (Xu et al., 2012).

Currently most knowledge on the role of plant APC/C E3 ligases was gained from investigating loss- and gain-of-function phenotypes of individual APC subunits. As mentioned, loss of APC2 results in aberrant megagametogenesis with the female gametophytes being arrested at the two-nucleus stage (Capron et al., 2003b). In addition, the corresponding mutants accumulate a D-box containing cyclin A3:GUS reporter in the embryo sacs (Capron et al., 2003b). Likewise, *apc4*, *apc6*, and *apc3*$^{cdc27a/hbt}$ double mutants are also affected in female gametogenesis, and for *apc4* and *apc6,* accumulation of a cycB1;1:GUS reporter in the embryo sacs was observed (Kwee and Sundaresan, 2003; Wang et al., 2012). Overall these findings demonstrate the importance of the plant APC/C complex for gametogenesis, and provide strong evidence that the E3 ligase confers degradation of mitotic cyclins.

CDC27a and HBT, the two APC3 homologs encoded in the Arabidopsis genome, are to some degree functionally redundant. Viable single null mutants can fully progress through gametogenesis and embryogenesis, but loss of both genes is lethal for the plant (Willemsen et al., 1998; Blilou et al., 2002; Perez-Perez et al., 2008). While *cdc27a* null mutants are indistinguishable from wild type, *hbt* mutants display a strong dwarf phenotype and reduced auxin sensitivity that is connected with a reduced root elongation zone and compromised cell divisions in leaves (Blilou et al., 2002; Perez-Perez et al., 2008). Similarly *apc6 and 10* hypomorphic mutants showed an overall reduced shoot habit and

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aberrant architecture of the vascular tissue with smaller epidermal leaf cells (Marrocco et al., 2009).

Of note is that the analysis of leaf cells from *hbt*, *apc6* and *apc10* mutants detected changes in the DNA ploidy levels when compared to wild type cells; the mutants had less cells with 8C and 16C DNA contents, while cells with 2C and 4C DNA contents were found in higher frequencies (Serralbo et al., 2006; John and Qi, 2008; Perez-Perez et al., 2008; Marrocco et al., 2009). These results are in agreement with earlier works on *ccs52a* mutants, which also reported aberrant DNA contents (Cebolla et al., 1999; Vinardell et al., 2003). The lower ploidy levels in these different mutants indicate that the corresponding cells less frequently undergo endoreduplications, an alternative DNA replication cycle – or endocycle - which is not interceded by mitosis or cytokinesis, and thus assigns the APC/C complex a function in endoreduplication onset in leaves.

CCS52 genes were in fact first described in *Medicago sativa* nodules, where their high expression levels correlated with differentiating cells undergoing endoreduplication (Cebolla et al., 1999), and Arabidopsis *CCS52A2 (At4g11920)* shows highest levels of expression when leaf cells exit mitosis and may undergo a mitosis-to-endocycle transition. Consequently, ectopic expression of *CCS52A2* results in leaf cells prematurely progressing into endocycles (Lammens et al., 2008).

CCS52A1 (At4g22910) is also expressed in the root elongation zone, while *CCS52A2* expression is restricted to the distal region of the root meristem (Vanstraelen et al., 2009). Loss of *CCS52A1* results in larger meristems with longer cells and primary roots when compared to wild type (Vanstraelen et al., 2009). Remarkably, the opposite effect is observed in *ccs52a2* null mutants, which have smaller root meristems and shorter roots then wild type, while ploidy levels are comparable to wild type (Vanstraelen et al., 2009). These findings indicate that the two APC/C co-activators have different modes of function in the root: while APC/$C^{CCS52A1}$ is discussed to control root meristem size through stimulating endoreduplication cycles in the elongation zone, APC/$C^{CCS52A2}$ is thought to act directly on the quiescent center and stem cells to regulate maintenance and structure of the root meristem (Vanstraelen et al., 2009). However, their different roles are based on their individual expression patterns rather than on different protein properties as CCS52A1 can complement the *ccs52a2* null mutant phenotype when expressed under control of the CCS52A2 promoter (Vanstraelen et al., 2009).

In contrast to CCS52A proteins, little is known about the function of CCS52B (At5g13840). This co-activator appears to also function in endocycle control since overexpression of *Medicago truncatula CCS52B* in tobacco BY2 suspension culture cells results in increased numbers of cells with a 4C DNA content compared to control cells that remain predominantly in a 2C state (Tarayre et al., 2004).

An interesting connection between *CCS52A* genes and nematode infection has been recently recognized where the biotropic pathogen appears to take over the host's cell cycle control machinery (de Almeida Engler et al., 2012; Vieira et al., 2013). Root-knot and cyst nematodes induce galls and syncytia in their hosts that consist of multinucleated giant-feeding cells with enlarged nuclei and higher DNA polyploidy levels. They represent critical feeding site for the pathogens, and analysis of these syncytia in infected Arabidopsis plants showed strongly up-regulated expressions of *CCS52A1* and *CCS52B* (de Almeida Engler et al., 2012). Ectopic overexpression of *CCS52B* further increased the nematode infection rate and syncytia development, while down-regulation of *CCS52* genes overall negatively impacted these processes (de Almeida Engler et al., 2012).

Analysis of the five *CDC20* members from Arabidopsis showed that the expression of *CDC20.1 (At4g33270)* and *20.2 (At4g33260)* begin to rise in S-phase and to peak at late M-phase before they drop again (Kevei et al., 2011). The other three *CDC20* members appear not to be expressed at any phase in the cell cycle or in different tissues tested (flowers, cauline and rosette leaves, stems and roots), leaving it currently unclear whether they are potential pseudogenes (Kevei et al., 2011). Yeast-two hybrid studies further showed that CDC20.1 and 20.2 interact with the APC10 core subunit and the potential substrates CYCA1;2 (At1g77390), CYCB2;1 (At2g17620), and CYCB2;2 (At4g35620), underscoring their function as co-activators in a plant APC/C holoenzyme and as cell cycle regulators (Kevei et al., 2011). This is further supported by their expression profiles since both genes are expressed in mitotically active tissues such as the root meristematic zone and pollen, and their down regulation by RNA interference causes male sterility and shorter roots (Kevei et al., 2011). CDC20.1 and 20.2 also assemble with all members of the CCS52 family, which allows for potentially similar roles as described for their animal counterparts to control each other's stability as part of an APC/C complex.

Finally, a largely unknown factor in plant APC/C function is the mechanisms controlling activity of the complex. So far described are *ULTRAVIOLET-B-INSENSITIVE4* (*UVI4; At2g42260*) and its homolog *OMISSION OF SECOND DIVISION1/GIGAS CELL* (*OSD1/GIGAS; At3g57860*) (Hase et al., 2006; Van Leene et al., 2010; Iwata et al., 2011). *UVI4* was originally identified in a screen for UV-B resistant mutants, and loss of *UVI4* further leads to increased endoreduplication rates (Hase et al., 2006). *osd1* null mutants are affected in the meiosis of male and female gametogenesis where they generate viable diploid gametes, allowing the rise of tetraploid offspring (d'Erfurth et al., 2010). Both proteins are considered to be negative regulators of APC/C complex activities, likely by their assembly with the CDC20 and CCSA52 co-activator subunits, and their loss negatively affects stability of the mitotic A-type cyclins (d'Erfurth et al., 2010; Heyman et al., 2011; Iwata et al., 2011). However their exact roles within the cell cycle still remain to be resolved.

REGULATION OF CRLS

With the exception of APC/C, CRL enzymes are regulated by the dynamic modification of the cullin subunit by NEDD8/RUB. NEDD8 is a ubiquitin-like modifier (UBL) sharing ~ 60% identity to ubiquitin that is found throughout eukaryotes and is conjugated to lysine residues of substrate proteins via an E1-E2-E3 cascade analogous to ubiquitylation. Conjugation of NEDD8 (neddylation) to a conserved C-terminal domain lysine of cullins greatly stimulates CRL ubiquitylation activity by promoting structural changes in the cullin and RBX1 subunits that both promote E2 recruitment and are believed to reposition the RBX1-tethered E2 enzyme into a closer proximity to the CRL ubiquitylation substrate (Duda et

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al., 2008; Saha and Deshaies, 2008). Neddylation of cullins also precludes binding of the CULLIN-ASSOCIATED NEDDYLATION DISSOCIATED (CAND1; At2g02560) protein to cullins. As detailed below, CAND1 and substrate adaptors bind to the cullin-RBX1 CRL core in a mutually exclusive manner.

Plant genomes contain multiple genes encoding NEDD8. In the case of Arabidopsis, the *RUB1* and *RUB2* (At2g35635) genes encode nearly identical ubiquitin-NEDD8 fusion proteins while *RUB3* (At1g11980) encodes a monomeric NEDD8 protein that is more divergent (Rao-Naik et al., 1998). Like ubiquitin, these NEDD8 precursor proteins must be processed to remove short C-terminal extensions to expose the C-terminal glycine residue needed for substrate conjugation. Furthermore, in the case of RUB1 and RUB2, the ubiquitin moiety must be removed to release monomeric NEDD8. While multiple NEDD8 processing enzymes have been identified in yeast and animals (recently reviewed in Mergner and Schwechheimer, 2014), the hydrolases from plants responsible for NEDD8 processing remain to be identified. Analyses of Arabidopsis T-DNA insertion mutants of *RUB1* and *RUB2* detected no apparent phenotype. However, *rub1 rub2* double mutants exhibit an early embryonic arrest phenotype similar to *cul1* null mutants (Bostick et al., 2004). Thus, as in most other eukaryotes, NEDD8 is essential for viability.

The NEDD8 conjugation pathway

Like ubiquitin, NEDD8 conjugation involves an E1-E2-E3 enzymatic cascade. In Arabidopsis, the AUXIN RESISTANT1 (AXR1; At1g05180) and E1 C-TERMINAL RELATED1 (ECR1; At5g19180) proteins function as a bipartite NEDD8 E1 enzyme (del Pozo et al., 1998; del Pozo et al., 2002b). Once activated, NEDD8 is transferred to the RUB CONJUGATING ENZYME1 (RCE1; At4g36800) E2 enzyme (del Pozo and Estelle, 1999). The RBX1 CRL subunit interacts with RCE1 and cullins and likely functions as a NEDD8 ligase. Consistent with this possibility, overexpression of RBX1 dramatically increased the abundance of neddylated CUL1 (Gray et al., 2002), whereas CUL1 neddylation was diminished in RBX1 knock-down seedlings (Lechner et al., 2002). In animals and yeast, the DEFECTIVE IN CULLIN NEDDYLATION1 (DCN1) protein has also been proposed to function as a NEDD8 E3 ligase (Kurz et al., 2005; Kurz et al., 2008). DCN1 binds to cullins and RBX1 (Yang et al., 2007; Kurz et al., 2008). However, DCN1 contains neither a RING nor a HECT (Homologous to the E6-AP Carboxyl Terminus) domain, one of which is present in all known ubiquitin and UBL ligases. Recent structural work suggests that DCN1 and RBX1 function synergistically as a dual NEDD8 ligase, with DCN1 enhancing the substrate specificity of RBX1-bound RCE toward cullins (Scott et al., 2010).

The identification and analysis of auxin response mutants has been instrumental in elucidating the regulation of CRL activity by the NEDD8 modification pathway. As previously discussed, auxin response requires the SCF$^{TIR1/AFB}$ ubiquitin-ligases. The auxin-resistant root growth phenotype conferred by mutations that diminish SCF$^{TIR1/AFB}$ activity has facilitated simple, yet powerful genetic screens for identifying components and regulators of this CRL, including genes encoding neddylation enzymes. Mutations in *AXR1*, the first cloned auxin response locus, confer severe defects in auxin sensitivity as a result of reduced SCF$^{TIR1/AFB}$ activity and the stabilization of Aux/IAA proteins (Lincoln et al., 1990; Leyser et al., 1993; Gray et al., 2001). The *axr1* mutant phenotype is highly pleiotropic however, as the activities of many additional CRLs are also predicted to be diminished as a result of reduced cullin neddylation. For example, *axr1* seedlings also exhibit resistance to cytokinin, ethylene, ABA, and brassinosteroids, and display reductions in ethylene biosynthesis and cold-responsive gene expression (Timpte et al., 1995; Schwechheimer et al., 2002; Tiryaki and Staswick, 2002). Likewise mutations in *ECR1* (del Pozo et al., 2002b; Woodward et al., 2007) and *RCE1* (Dharmasiri et al., 2003; Larsen and Cancel, 2004), as well as over- or under-expression of *RBX1* (Gray et al., 2002; Lechner et al., 2002; Schwechheimer et al., 2002) also confer related pleiotropic phenotypes, including severe defects in auxin response.

Reductions in *AXR1*, *ECR1*, *RCE1*, or *RBX1* function all result in a slight reduction in cullin neddylation (Lechner et al., 2002; Dharmasiri et al., 2003; Woodward et al., 2007). However, none of these mutants or transgenics confer the early embryo arrest phenotype characteristic of *rub1 rub2* or *cul1-null* mutants. This is likely due to a combination of the residual function of hypomorphic alleles and genetic redundancy. The Arabidopsis genome contains single closely related paralogs of *AXR1*, *RCE1*, and *RBX1*. Characterization of the *AXR1* paralog, *AXR1-LIKE* (*AXL; AT2g32410*) confirmed that AXL also functions as a NEDD8 activating enzyme subunit, and *axr1 axl-1* double mutants exhibit an embryo or early seedling-lethal phenotype and are severely impaired in CUL1 neddylation (Dharmasiri et al., 2007). Curiously however, although overexpression of *AXL* in *axr1-3* plants was able to complement the phenotypes associated with this weak *axr1* mutant, when expressed from the *AXR1* promoter *AXR1* and *AXL* differed in their ability to complement the severe *axr1-30* mutation suggesting that some functional diversity may exist *in vivo* (Hotton et al., 2011).

To date, the possible involvement of DCN1 in cullin neddylation has not been rigorously examined in plants. Genes encoding DCN1 and DCN1-like proteins are present in plant genomes, and mutation of the Arabidopsis *DCN1-like* gene *ANTI-AUXIN RESISTANT3* (*AAR3; At3g28970*) confers resistance to the synthetic auxin 2,4-D (Biswas et al. 2007). However, direct assessments of cullin neddylation status have not yet been reported for *aar3* mutants. Additionally, a second recent study found that RNAi knock-down of a putative *DCN1* homolog in tobacco (NtDCN1) impaired pollen development and zygotic embryogenesis (Hosp et al. 2014). NtDCN1 can interact with both CUL1 and NEDD8 *in vitro*, but its importance in cullin neddylation remains to be established.

Thus far, Arabidopsis remains the only plant in which the NEDD8 pathway has been rigorously characterized. In large part, this is due to the absence of NEDD8 conjugation pathway mutants in other plant species. However, the recent identification of the neddylation inhibitor MLN4924 provides a pharmacological approach toward addressing NEDD8-mediated regulation (Soucy et al., 2009). MLN4924 was initially identified as an inhibitor of the human NEDD8 activating enzyme, and is a promising anti-cancer drug currently undergoing clinical trials (for a discussion on these trials see Soucy et al 2009 and Zhao et al 2012). The activating enzyme catalyzes formation of a NEDD8-MLN4924

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adduct resembling the normal conjugation intermediate, NEDD8-adenylate, and stably binds to the active site of the activating enzyme to block activity (Brownell et al., 2010). Recently, MLN4924 was found to be an effective neddylation inhibitor in Arabidopsis as well as several additional species (Hakenjos et al., 2011), thus providing a powerful new tool for probing CRL regulation by neddylation in diverse plant species.

The COP9 signalosome (CSN) functions as a NEDD8 isopeptidase

The regulation of CRL activity by NEDD8 modification of the cullin subunit is highly dynamic. NEDD8 is removed from cullins (termed deneddylation or deconjugation) by the COP9 signalosome (CSN). The CSN complex is found throughout eukarytotes and consists of eight subunits (CSN1-8). It was originally identified in Arabidopsis through genetic screens for mutants exhibiting a *c*onstitutive *p*hotomorphogenic/*de*-*et*iolated *(cop/det)* phenotype, and was subsequently biochemically purified from both plant and animal protein extracts (Wei et al., 1994; Chamovitz et al., 1996). The CSN is structurally related to the lid of 19S regulatory particle of 26S proteasome and eukaryotic translation initiation factor 3 (eIF3), and is composed of six PCI (Proteasome, COP9, eIF3) domain-containing subunits (CSN1-4, CSN7, and CSN8) and two MPN (Mpr1p, Pad1p N-terminal) domain-containing subunits (CSN5 and CSN6) (Wei et al., 1994). Arabidopsis contains single genes encoding each of the PCI subunits, while the two MPN subunits are encoded by pairs of closely related genes (*CSN5a/CSN5b (At1g22920/At1g71230)* and *CSN6a/CSN6b (At5g56280/At4g26430)*). The CSN binds CRLs by interacting with cullin and RBX1 through the CSN2 and CSN6 subunits (Schwechheimer et al., 2001; Enchev et al., 2012). Cullin deneddylation is catalyzed by the JAMM metallozyme motif within the CSN5 subunit (Lyapina et al., 2001; Cope et al., 2002). However, CSN5 can only provide this isopeptidase activity after incorporation into the CSN holocomplex, as monomeric CSN5 is not an active deneddylase *in vitro*, and deneddylation activity is defective *in planta* in all CSN subunit mutants (Dohmann et al., 2005; Gusmaroli et al., 2007; Dohmann et al., 2008a).

As discussed above, neddylation promotes CRL activity. Therefore, since the CSN removes NEDD8 from cullins, the CSN would be expected to function as a negative CRL regulator. Furthermore, at least in the case of SCF complexes, CSN binding prevents both CUL1 (re)-neddylation and interactions with E2 enzymes, thus providing additional negative regulatory actions (Enchev et al., 2012). Indeed, the addition of purified CSN to *in vitro* CRL ubiquitylation assays results in reductions in both cullin neddylation and substrate ubiquitylation (Cope et al., 2002; Yang et al., 2002). However, genetic studies have clearly demonstrated that CSN function is required for optimal CRL activity *in vivo*. In Arabidopsis, null mutants of any CSN subunit are phenotypically indistinguishable and display the pleiotropic *cop/det/fus* phenotype, characterized by the short hypocotyl and open cotyledon phenotypes of dark-grown seedlings, accumulation of anthocyanin and seedling lethality (Wei et al., 1994; Serino et al., 1999; Gusmaroli et al., 2007). As discussed above, this is the result of reduced activity of the CUL4-based COP1-SPA E3 ligase, and stabilization of HY5 and additional light-regulated transcription factors (Chen et al., 2006; Chen et al., 2010; Lau and Deng, 2012). Additionally, analyses of viable hypomorphic *csn* mutant/transgenic plants have detected defects in additional CRL-regulated pathways including auxin (Schwechheimer et al., 2001; Dohmann et al., 2008a; Zhang et al., 2008a; Stuttmann et al., 2009; Huang et al., 2013a), JA (Feng et al., 2003), GA (Dohmann et al., 2010), and SA (Spoel et al., 2009) signaling, cold response (Schwechheimer et al., 2002), cell cycle regulation (Dohmann et al., 2008b), and flower development (Wang et al., 2003). In the case of auxin, GA, and SA signaling, CRL substrates have been found to be stabilized as a result of reduced CSN function. Thus, the pleiotropic *csn* mutant phenotype is likely due to the impairment of hundreds of CRL ligases.

The basis for the paradox of the negative regulatory role of the CSN on CRL activity observed *in vitro* and the demonstrated positive regulatory role obtained through genetic studies of *csn* mutants remains unclear. In yeast and animal systems, CSN-mediated deneddylation has been found to protect the cullin (Wu et al., 2005), and some substrate adaptors from autoubiquitylation (Cope et al., 2002; Zhou et al., 2003; Wee et al., 2005; Schmidt et al., 2009). This is thought to be due to the combined effects of deneddylation lowering CRL activity and CSN-associated deubiquitylating enzymes that remove ubiquitin molecules that have been autocatalytically attached to CRL subunits. Thus, the CRL-related defects of *csn* mutants are likely due at least in part to a reduction in CRL subunit abundance. However, this possibility has not been rigorously examined in plants. Steady-state level of transgenically expressed TIR1 was slightly reduced in *csn2-5 sgt1b* double mutants compared to wild-type and *sgt1b* single mutants (Stuttmann et al., 2009). TIR1 abundance in *csn2-5* single mutants was not examined however, precluding a clear interpretation as to whether or not reduced CSN function alone might be sufficient to destabilize TIR1. More recently, the F-box protein, COP9 INTERACTING F-BOX KELCH (CFK1; At5g432350), was shown to exhibit reduced stability in *csn4* mutant seedlings (Franciosini et al., 2013). In the case of cullins, CUL3 levels are diminished in *csn* mutants, but CUL4 abundance is increased while CUL1 levels appear unchanged (Gusmaroli et al., 2007). Clearly more work is required to determine to what extent reductions in CRL subunit stability might contribute to the diminished CRL activity of *csn* mutants in plants. However, together with studies of NEDD8 conjugation mutants, these findings clearly indicate that neddylation of cullins is highly dynamic, with both conjugation and cleavage being required for optimal CRL function.

As discussed earlier in this chapter, pathogens frequently exploit CRLs to promote pathogenesis. This extends to the NEDD8 pathway. The Epstein-Barr human herpesvirus encodes a deneddylase activity (BPLF1) capable of removing NEDD8 from cullins (Gastaldello et al., 2010). As expected, expression of BPLF1 alone or in the context of viral infection results in reduced CRL activity and corresponding cell cycle defects. Importantly, the deneddylase activity of BPLF1 is required for viral DNA replication, as it leads to the accumulation of the Cdt1 licensing factor, a substrate of both SCF^{Skp2} and CRL4-$DDB1^{Cdt2}$. In plants, the geminivirus C2 protein interacts with the catalytic CSN5 subunit of the CSN and inhibits deneddylation of CUL1 (Lozano-Duran et al., 2011). Expression of C2 in Arabidopsis confers weak *csn*-like phenotypes including reduced response to auxin, JA, and GA.

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Although SCF^{COI1} activity was not assessed directly in this study, the C2-mediated suppression of JA response may be of particular importance to the virus, as methyl-JA (MeJA) treatment of Arabidopsis reduces viral infection.

CAND1-mediated cycling of CRL substrate adaptors

The Cand1 protein was identified as a cullin binding protein in animal cells (Liu et al., 2002; Zheng et al., 2002a). Initial biochemical studies revealed that Cand1 specifically binds to unneddylated cullins, forming a ternary complex with the cullin-Rbx1 catalytic core. Since Cand1 binding to cullin-Rbx1 precludes substrate adaptor binding, Cand1 was proposed to negatively regulate CRL activity by preventing CRL assembly. Structural studies of the Cand1-Cul1-Rbx1 ternary complex provided support for this model, as Cand1 binding blocks both the substrate adaptor binding site and the neddylation site (Goldenberg et al., 2004). However, as with the CSN complex, genetic studies in plants revealed that *cand1* mutants paradoxically exhibit reduced CRL activity. *cand1* mutants were isolated in forward genetic screens for auxin resistant root growth (Cheng et al., 2004; Chuang et al., 2004), defects in leaf vein patterning in Arabidopsis (Alonso-Peral et al., 2006), and impaired crown root emergence in rice (Wang et al., 2011). T-DNA null alleles of *cand1* have also been characterized (Feng et al., 2004). Like *csn* and NEDD8 pathway mutants, the *cand1* mutant phenotype is highly pleiotropic. *CAND1* is not essential for viability however. Multiple CRL substrates, including RGA, HY5, and Aux/IAA proteins are stabilized in *cand1* mutants (Chuang et al., 2004; Feng et al., 2004), demonstrating that CAND1 promotes CRL activity *in vivo*. This reduction in CRL activity is not due to changes in cullin neddylation however, as cullin neddylation status is unaltered in *cand1* mutants. Loss of CAND1 function dramatically enhances the modest *cop* phenotype of *csn1-10*, a weak CSN subunit mutant (Zhang et al., 2008a). Again however, this appears to occur independently of changes in cullin neddylation status.

To reconcile the conflicting biochemical studies indicating that CAND1 negatively regulates CRL activity with the above genetic findings from plant *cand1* mutants, a model was proposed whereby CAND1-mediated cycles of CRL assembly and disassembly are required for proper function *in vivo* (Cope and Deshaies, 2003). Following substrate ubiquitylation, the CSN removes NEDD8 from the cullin. Since CAND1 only binds unmodified cullin, CAND1 was proposed to promote CRL disassembly, with subsequent ubiquitylation events requiring dissociation of the cullin-RBX1 core subunits from CAND1, and re-assembly of the CRL by cullin-RBX1 binding new substrate adaptors. Thus, if cycling is required for normal CRL function, even mutations in factors promoting disassembly such as CAND1, would disrupt the cycle and reduce ubiquitin-ligase activity. This cycling model has been addressed within the context of both CUL1- and CUL3-based CRLs. In animal cells, association of the BTB protein Keap1 with Cul3 was increased by siRNA-mediated knockdown of *Cand1* expression (Lo and Hannink, 2006). Likewise in Arabidopsis, loss of *cand1* function results in a significant increase in the proportion of TIR1-ASK substrate adaptor that is assembled into SCF^{TIR1} complexes (Zhang et al., 2008a). Both of these studies support the notion that CAND1 functions as a CRL disassembly factor with CRL assembly/disassembly cycles. Furthermore, this cycling is essential for optimal CRL activity, as CRL3^{Keap1} and SCF^{TIR1} substrates were stabilized as a result of reduced CAND1 activity despite the fact that the abundance of assembled CRL3^{Keap1} and SCF^{TIR1} complexes was increased.

Complementing the above study on SCF^{TIR1} assembly in *cand1* mutants was analysis of the *axr6-2* mutant allele of CUL1 (Hobbie et al., 2000). *axr6-2* is a semi-dominant missense mutation located near the N-terminus of CUL1 in a region that contacts both SKP1 and CAND1 (Zheng et al., 2002b; Hellmann et al., 2003; Goldenberg et al., 2004). *AXR6/axr6-2* plants exhibit modest auxin resistance phenotypes, whereas *axr6-2* homozygotes fail to develop basal embryonic structures and display seedling lethality. Interestingly, this missense mutation results in a dramatic increase in CAND1-CUL1 complex abundance and a corresponding reduction in assembled SCF complex (Zhang et al., 2008a), suggesting that *axr6-2* causes a block at a distinct step of the SCF cycle – where CAND1 and CUL-RBX1 should normally dissociate to allow SCF reassembly. Genetic studies confirmed that the major defect of *axr6-2* is the inability to dissociate from CAND1, as *cand1* mutation suppressed the seedling lethal phenotype of *axr6-2* homozygotes, thus providing additional support for CAND1-mediated cycling of CRL complexes.

Why must CRL complexes undergo cycles of assembly and disassembly? For all CRLs, there is an abundance of substrate adaptors competing for access to the common cullin-RBX1 catalytic core. CRL cycling is likely a mechanism for ensuring that a cell's repertoire of assembled CRLs can adjust to varying substrate demands. Recent studies of SCF complexes in yeast and animal cells support this hypothesis. Schmidt et al. 2009 first proposed that CAND1 might function as a substrate adaptor exchange factor when they observed that *knd1* (*S. pombe CAND1*) mutants display imbalances in different SCF complexes. For example, whereas SCF^{Pof1} abundance was elevated in *knd1* mutants, SCF^{Pof3} abundance was diminished. More recently, multiple studies have confirmed that CAND1 does indeed function as a substrate adaptor exchange factor for SCF complexes and perhaps other CRLs (Pierce et al., 2013; Wu et al., 2013; Zemla et al., 2013). Employing a FRET-based approach to monitor association and dissociation kinetics, Pierce et al. 2013 demonstrated that Cand1 rapidly dislodges Skp1-F-box protein dimers from Cul1-Rbx1. Similarly, Skp1-FPB rapidly liberates Cul1-Rbx1 from Cand1. They then tested the exchange factor hypothesis directly in an elegant experiment where Skp1-F-box/WD repeat-containing protein 7 (Fbxw7) alone or a mixture of Skp1-Fbxw7 and Cand1 were added to preformed SCF$^{\beta\text{-TrCP}}$ complexes and ubiquitylation of the SCF^{Fbxw7} substrate cyclin E was assessed. Whereas addition of Skp1-Fbxw7 alone was unable to assemble an SCF^{Fbxw7} complex and ubiquitylate cyclin E, the addition of Skp1-Fbxw7 and Cand1 together resulted in robust cyclin E ubiquitylation, thus demonstrating that Cand1 can activate SCF complexes by providing new substrate adaptors access to the Cul1-Rbx1 catalytic core. This beautiful work identifying Cand1 as a CRL substrate adaptor exchange factor represents a major advance in our understanding of the complexities of CRL dynamics and regulation. Furthermore, since at least some substrate adaptors are highly dependent upon Cand1 for assembling into CRLs, it explains why these CRLs exhibit reduced activity in *cand1* mu-

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tants. However, it remains puzzling why those CRLs whose abundance is increased as a result of reduced Cand1 function, such as CRL3[Keap1] and SCF[TIR1], also exhibit reduced E3 activity.

Dynamic coordination between NEDD8 and CAND1 optimizes the CRL landscape

With the notable recent exception of carbon source regulation in yeast (Zemla et al., 2013), there is little evidence for regulation of cullin neddylation at the global level. However, cullin neddylation at the level of the individual CRL is highly regulated. *In vitro* experiments and studies from animal cells suggest that cullin neddylation status is controlled such that the cell's complement of active CRLs matches substrate demands. The addition of substrate adaptors and substrate to *in vitro* assays both promoted cullin neddylation (Bornstein et al., 2006), and inhibited CSN-mediated deneddylation (Emberley et al., 2012), suggesting that individual CRLs are activated by the presence of substrate (Figure 6). Once this substrate has been ubiquitylated and degraded, the CSN is then able to deneddylate the cullin and inactivate the CRL. Deneddylation then enables Cand1 to disassemble the CRL, and available substrate adaptors can displace Cand1 to assemble new CRLs. If no substrate is available, the nascent CRL returns to the Cand1 cycle. Alternatively, if substrate is present, it promotes cullin neddylation and inhibits CSN-mediated deneddylation, leading to CRL activation.

SUMMARY

Since their discovery in the mid 1990s, tremendous progress has been made in understanding the function of CRL E3 ligases (Deshaies 1999). As overviewed in this chapter, it appears that almost all aspects in plant biology, from development and physiological processes to stress responses, are tightly linked to the activity of

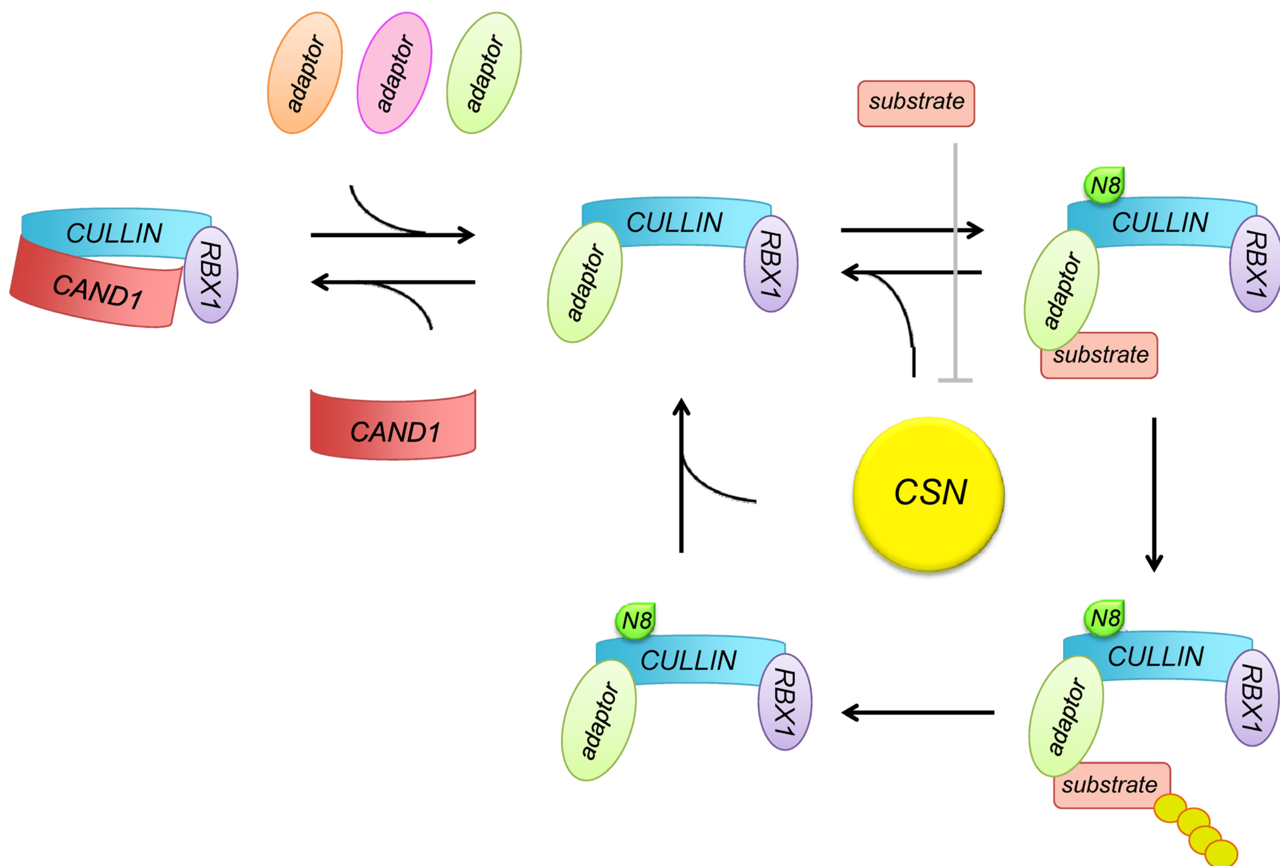

Figure 6. CRL regulation by CAND1 and NEDD8.

CAND1 functions as a substrate adaptor exchange factor, facilitating the formation of a dynamic cellular pool of CRLs. Once assembled, the cullin subunit is neddylated. If no substrate is available, the CRL is rapidly deneddylated, returning the CRL to the CAND1 cycle. Alternatively, the presence of substrate promotes CRL activity by inhibiting CSN-mediated deneddylation. Upon substrate depletion by the 26S proteasome, the CSN deneddylates the cullin and the CRL is disassembled by CAND1, enabling new CRLs to form. N8 = NEDD8.

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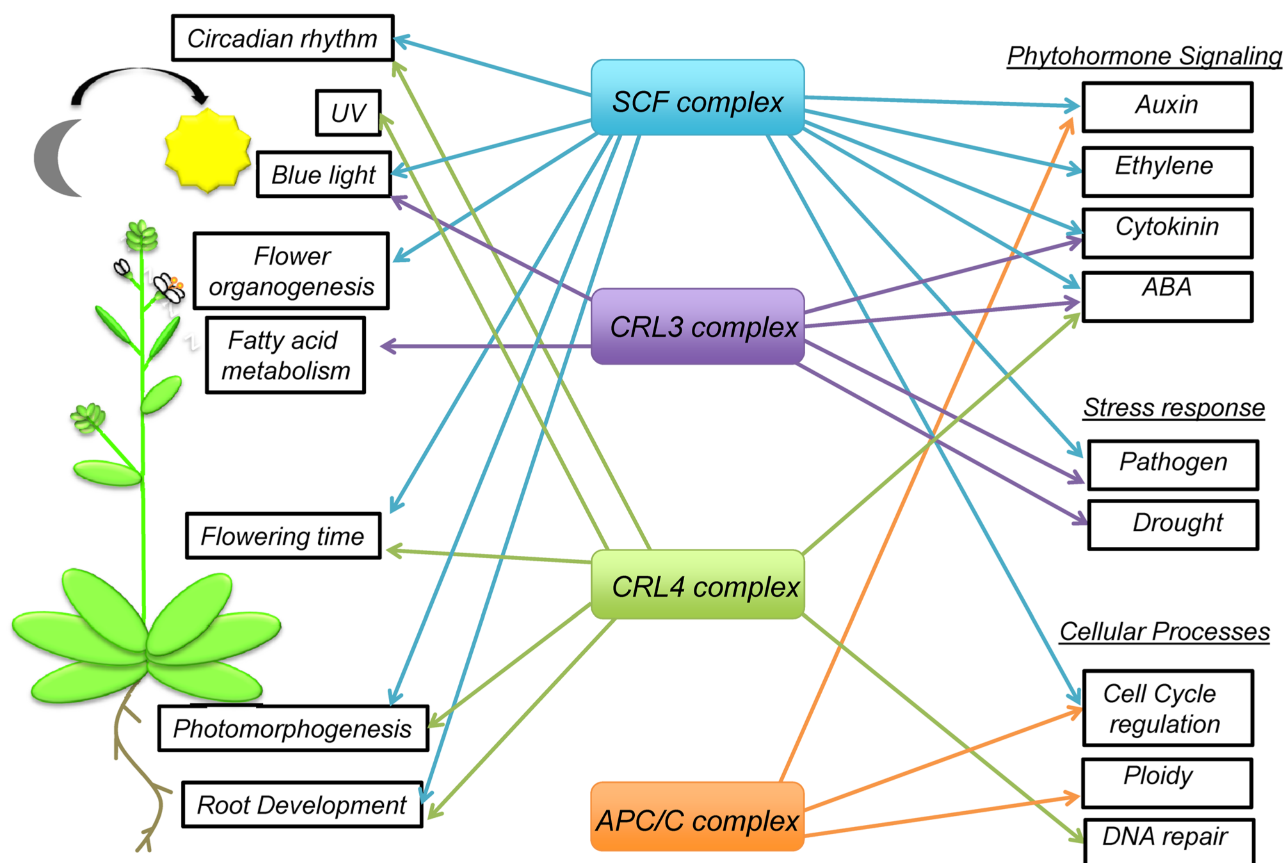

Figure 7. Overview of CRL complex functions in various plant processes.

these enzymes (Figure 7). This is reflected in the high number of substrate adaptors and target proteins that have evolved in plants.

There are several important ongoing areas of research, which will continue to broaden the understanding of E3 ligase activities. One is the identification of specific substrates. As this list increases so does the knowledge of the different cellular pathways in which they participate. A second important aspect will be to identify and characterize regulatory steps that control the activity of these different E3 ligase complexes. Major progress has been made over the last decade on how neddylation, CAND1, and the CSN complex impact CRL activity and assembly with substrates. Additionally the need for small molecular cofactors and secondary modifications of substrate adaptors, or their substrates, to trigger assembly into a CRL complex are being discovered. It is expected that in many cases such modifications, like binding of a phytohormone, phosphorylation, or additional adaptor proteins, are critical for efficient functioning of these ligases.

A third challenge is to unravel the existing cross-talk and integration between the different classes of CRL E3 ligases, and how this impacts their activities. It is striking that the four CRL classes discussed in this chapter frequently function in similar developmental or cellular processes. Understanding the mechanisms that the plant has in place to coordinate CRL activities will provide further insights into where these enzymes are positioned in the regulatory network.

In conclusion, despite the fact that many mechanistic aspects about the 26S ubiquitin proteasome pathway are now understood, and the importance of CRL E3 ligases in plants is quite clear, we are still at the beginning in defining the breadth of the pathway and the regulatory tools controlling their activity.

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