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CUL3 E3 ligases in plant development and environmental response

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Abstract

Thirty years of research have revealed the fundamental role of the ubiquitin–proteasome system in diverse aspects of cellular regulation in eukaryotes. The ubiquitin–protein ligases or E3s are central to the ubiquitin–proteasome system since they determine the specificity of ubiquitylation. The cullin–RING ligases (CRLs) constitute one large class of E3s that can be subdivided based on the cullin isoform and the substrate adapter. SCF complexes, composed of CUL1 and the SKP1/F-box protein substrate adapter, are perhaps the best characterized in plants. More recently, accumulating evidence has demonstrated the essential roles of CRL3 E3s, consisting of a CUL3 protein and a BTB/POZ substrate adaptor. In this Review, we describe the variety of CRL3s functioning in plants and the wide range of processes that they regulate. Furthermore, we illustrate how different classes of E3s may cooperate to regulate specific pathways or processes.

It is generally understood that the control of gene expression involves the regulation of translation and protein stability in addition to transcription. In fact, many recent studies have shown that the correlation between RNA transcript and protein levels is often weak or non-existent, indicating that protein stability, among other parameters, may be key to determining protein abundance[1]. The ubiquitin–proteasome system (UPS) has a central role in the regulation of protein stability. The system is highly conserved in eukaryotes and is crucial for many biological processes[2]. The ATP-dependent ubiquitin conjugation pathway involves ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin–protein ligase (E3) enzymes. Although ubiquitylation can have a number of effects, the most common is protein degradation via the 26S proteasome. Among these enzymes, E3s exhibit the most diversity and play a central role in the selectivity of ubiquitin-mediated protein degradation. In plants, the E3 ligases can be classified into four main types: HECT (homologous to the E6-AP carboxyl terminus); RING (really interesting new gene); U-box; and cullin–RING ligases (CRLs). The HECT, RING and U-box E3 ligases are single polypeptides, whereas the CRLs consist of multiple subunits[3,4]. In plants, CRLs are probably the best-characterized E3s to date, participating in almost all aspects of plant growth and development[4,5]. In

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the CRL complex, the cullin protein serves as an elongated scaffold, recruiting the RING-FINGER PROTEIN RING BOX PROTEIN 1 (RBX1) and E2 to its carboxy (C)-terminal region and binding a substrate adaptor to its amino (N)-terminal region. Four subtypes of CRLs are known in plants, each with a different cullin: CUL1, CUL3, CUL4 and the cullin-like protein ANAPHASE-PROMOTING COMPLEX 2 (APC2) (Fig. 1). CUL1 E3 ligases, also called SCF (S-PHASE KINASE-ASSOCIATED PROTEIN 1 (SKP1)–CUL1–F-box) complexes, are the most thoroughly studied class, in which the substrate adaptor consists of SKP1 and an F-box protein. Studies have shown that SCFs are crucial regulators in plant hormone responses, circadian rhythm, floral development and many other processes[4]. In recent years, CUL3 E3 ligases have also been identified as important regulators in numerous cellular processes in plants, such as plant hormone biosynthesis and signalling, light signalling and stress responses. This Review summarizes recent studies of CUL3 E3 ligases in plants and discusses their regulation and crosstalk with other E3 ligases (see Table 1 for a summary of the mentioned CUL3 E3 ligases). We also highlight many outstanding questions that await further investigation.

CUL3 E3 ligase subunits

Studies in *Caenorhabditis elegans* and humans were the first to identify CUL3-based E3 ligases. These studies showed that CUL3 interacts with substrate adaptors called BTB/POZ proteins (BROAD COMPLEX, TRAMTRACK, and BRIC-A-BRAC/POZ and ZINC FINGER), as well as RBX1, to form a new class of E3 ligases[6–8]. Subsequently, a diverse set of CUL3–BTB E3 ligases were reported in plants[9–12].

Similar to CUL1, CUL3 acts as a scaffold protein. There are two CUL3-encoding genes in *Arabidopsis thalania* (*CUL3a* and *CUL3b*) and three genes in *Oryza sativa* (*CUL3a*, *CUL3b* and *CUL3c*). In *Arabidopsis*, *cul3a/b* single mutants do not display obvious growth defects compared with wild-type plants, but double mutants are embryo lethal, indicating that CUL3a and CUL3b have overlapping functions and are essential[9,10,13]. *cul3a* single mutants exhibit a cell death phenotype in leaves and enhanced resistance to several rice pathogens[14].

RBX1 is a RING–H2 finger protein that binds the E2 ubiquitin-conjugating enzyme and brings it close to the E3 substrate. Two RBX1 proteins exist in *Arabidopsis* that share 83% sequence identity. One of them, RBX1a, is highly expressed in all tissues, and *rbx1* knockdown lines show severe defects in growth and development, indicating that RBX1 is essential in plants[15–17]. RBX1a was shown to interact with CUL1, CUL3 and CUL4, functioning as a core subunit in CRLs[9,11,15,16]. Two genes encode RBX1-like proteins in rice (*RBX1a* and *RBX1b*). They share 92.7% sequence identity and both interact with rice CUL3a[14].

The BTB/POZ proteins possess functional properties of both SKP1 and F-box proteins in SCF complexes[7]. All of the BTB/POZ proteins contain a conserved BTB/POZ domain, which interacts with CUL3. Protein analysis also indicates that, despite low sequence similarities in BTB/POZ proteins and SKP1, their BTB/POZ folds are structurally well conserved, consistent with their shared ability to bind cullins[18,19]. BTB/POZ

proteins usually contain additional protein–protein interaction domains, such as ankyrin, tetratricopeptide repeat (TPR), and MEPRIN AND TUMOUR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTOR HOMOLOGY (MATH) domains, which bind to substrates[7,20–22]. There are 80 and 149 BTB domain-containing proteins in *Arabidopsis* and rice respectively, classified into several subfamilies according to their additional domains[23] (see Fig. 2 for the phylogenetic tree of *Arabidopsis* BTB/POZ proteins). Of the BTB/POZ proteins tested, the majority, but not all, interact with CUL3, indicating that many of these proteins participate in CRL3 complexes[9–11].

Six BTB/POZ proteins in *Arabidopsis* are known as BTB/POZ–MATH 1–6 (BPM1–BPM6). BPMs contain a MATH domain within their N-terminal region responsible for binding substrates and a BTB/POZ domain in their C-terminal region. BPMs in *Arabidopsis* interact with and regulate the turnover of various transcription factors that play diverse roles in plant development[24–32]. It is noteworthy that a large-scale expansion and diversification of the MATH–BTB family occurred in grass species, with 6 members in *Arabidopsis*, 4 in alfalfa, 5 in tomato, 10 in *Brassica rapa*, 31 in maize and 68 in rice[23,33–36]. Expansion of this family in rice accounts for most of the difference in size of the BTB family compared with *Arabidopsis*. The analysis of different land plant species revealed that the MATH–BTB family can be divided into two groups: the core group that is common to most plant genomes examined; and the expanded group that was generated by local gene duplications in the grass lineage[23]. All of the MATH–BTB genes from banana, *Arabidopsis*, grapevine, *Selaginella* and *Physcomitrella*, together with some MATH–BTB genes in grasses (rice, sorghum, wheat and maize) belong to the core group[23,34]. The remaining genes in grasses are clustered into the expanded group, suggesting that they possess grass-specific functions[23,34]. MATH-BTB 1 (MAB1) in maize *Zea mays* and MAB2 in wheat *Triticum aestivum* proteins in the expanded group—both regulate embryogenesis and assemble with CUL3, indicating that the expanded-group proteins also function as substrate adaptors for CRL3s[33,37]. Interestingly, maize MAB1 and two homologues (MAB2 and MAB3), together with three triticum MAB homologues (MAB1, MAB2 and MAB3) are apparently germline-specific or embryo-specific genes, and the rice expanded MATH–BTB genes are not abundant in the rice RNA sequencing databases[34,38–40]. These results suggest that the expanded-group genes are expressed either at lower levels or in highly specific temporal or spatial patterns. Nevertheless, more proteins in the expanded MATH–BTB group need to be characterized to make their roles clear.

There are a number of additional families of BTB/POZ proteins. BTB–NPH3 proteins, also called NPH3/RPT2-like (NRLs), are plant-specific BTB/POZ proteins[18]. NRLs contain an N-terminal BTB domain and a C-terminal NON-PHOTOTROPHIC HYPOCOTYL 3 (NPH3) domain, and some members contain an additional C-terminal coiled-coil domain. There are 31 NRL family genes in *Arabidopsis*, one of which (AT3G49900) is not classified in the NPH3 clade according to our phylogenetic tree. Several of them have been implicated in phototropism, root gravitropism, chloroplast accumulation response and auxin-mediated plant development[41–45]. The BTB–ankyrin family exists in both metazoans and higher plants. Family members contain an N-terminal BTB domain and a C-terminal ankyrin repeat domain that is typically considered a protein–protein interaction motif, as well as a nuclear localization sequence[21]. Six BTB–ankyrin proteins are in

Arabidopsis, including NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEIN 1/2/3/4 (NPR1/2/3/4) and BLADE-ON-PETIOLE1/2 (BOP1/2). NPR1/3/4 play key roles in plant immunity, while BOP1/2 regulate leaf and flower development, photomorphogenesis and thermomorphogenesis[46–48]. BTB–TPR proteins are characterized by the presence of an N-terminal BTB domain and six TPR motifs, together with a coiled-coil motif in the C terminus. BTB–TPR proteins are only present in the plant kingdom, and *Arabidopsis* has three members, ETHYLENE OVERPRODUCTION PROTEIN 1 (ETO1) and ETHYLENE OVERPRODUCER-LIKE 1/2 (EOL1/2), which function in ethylene biosynthesis[49,50]. Another subfamily called light-response BTBs (LRBs) are also plant specific. They usually contain a putative nuclear localization sequence near the N terminus, followed by a BTB domain and then a BTB and C-terminal Kelch (BACK) domain near the C terminus. There are three LRBs in *Arabidopsis*, of which LRB1 and LRB2 play key roles in flowering and photomorphogenesis[51–53]. BTB–TAZ proteins contain an N-terminal BTB domain, a transcriptional adapter zinc finger (TAZ) domain and a C-terminal calmodulin (CaM)-binding domain. There are five BTB–TAZ members in *Arabidopsis* that have been shown to function in gametophyte development, auxin response, abscisic acid (ABA) response and stress responses[54,55]. Although the biochemical function of these five BTB–TAZ members has not been reported, apple (*Malus domestica*) BTB–TAZ 1/2 (BT1/2) can form active E3 ligase complexes[56]. BTB–Armadillo (ARM) proteins contain N-terminal ARM repeats and a C-terminal BTB domain. There are two members in *Arabidopsis*, named ARM-repeat protein interacting with ARM REPEAT PROTEIN INTERACTING with ABF2 (ARIA) and ARM BTB *Arabidopsis* protein 1 (ABAP1). ARIA functions in ABA signalling and stress tolerance, while ABAP1 negatively regulates DNA replication and cell proliferation during plant leaf development[57,58]. However, so far, these BTB–ARM proteins have not been shown to assemble with CUL3. Other BTB/POZ proteins, such as those with a pentapeptide domain or an F5/8-type C domain and those solely consisting of one or two BTB domains, are not well characterized.

CRL3s are involved in diverse processes in plants

In mammals, CRL3s play important roles in embryo development, mitotic progression, protein trafficking, developmental signalling and stress responses (ref.[59]). Research in recent years also established indispensable roles for CRL3s in plant development.

Embryogenesis and post-embryonic development.

Genetic studies show that *cul3a cul3b* double mutations in *Arabidopsis* affect embryo and endosperm development, resulting in embryo lethality[9,10,13]. Mutants of MAB1 in maize show defects in meiosis and mitosis, leading to an arrest of germline development[33]. Furthermore, Triticum MAB2 is also implicated in the regulation of translation initiation during the onset of embryogenesis, with EUKARYOTIC INITIATION FACTOR 3/4 (eIF3/4) as candidate substrates[37].

Besides embryogenesis, CRL3s also function in other developmental processes, such as flowering. BOP1 and BOP2, which belong to the BTB–ankyrin family, serve as substrate adaptors for CUL3-based E3 ligases and contribute to floral meristem formation

together with the transcription factor LEAFY (LFY)[48]. Paradoxically, BOP1/2 promote ubiquitylation of LFY in vitro but are required for LFY stability in vivo. LFY activity is also regulated by an SCF-type E3 called SCF^{UFO} (ref.[60]). How these two E3 ligases regulate LFY function is unknown but presents an interesting example of interaction between two types of CRL. BPM adaptors target and mediate the degradation of MYB56, which is a negative regulator of flowering[24]. BPMs positively affect flowering time, as evidenced by late flowering in *bpm* mutants[24] and early flowering in BPM1 overexpression lines[25]. A CRL3 is also involved in plant responses to vernalization. FRIGIDA (FRI) acts as a scaffold protein in a transcription activator complex to activate the expression of flowering time-related genes. LRB1 and LRB2 assemble with CUL3a and FRI to promote FRI degradation after cold stress. Furthermore, cold-induced WRKY34 transcription factors enhance CUL3a accumulation, which promotes FRI proteolysis[52].

Hormone biosynthesis and signalling.

The first demonstration of CRL3s in plants was the discovery of $CUL3^{ETO1}$ in *Arabidopsis*. This E3 ligase targets the enzyme 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 5 (ACS5) for degradation, thus negatively regulating ethylene synthesis[49]. ACS5, a type 2 ACS, is a rate-limiting enzyme in ethylene biosynthesis and converts *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid. ETO1 acts on ACS5 in two distinct ways. It inhibits ACS5 activity by directly binding the enzyme, and also promotes ACS5 degradation via an interaction with CUL3 (ref.[49]). In addition, enhanced ethylene production and ACS5 protein levels in *cul3a/b* knockdown mutants confirmed the regulation of ethylene biosynthesis by $CUL3^{ETO1}$ (ref.[61]). The homologues of ETO1, EOL1 and EOL2 were later shown to work along with ETO1, negatively regulating ethylene synthesis by promoting the degradation of type 2 ACSs[50].

A series of reports shed light on the important roles of CRL3s in ABA signalling. The six BPMs in *Arabidopsis* interact with HOMEOBOX 6 (HB6), a class I homeodomain–leucine zipper transcription factor, which acts as a negative regulator of ABA responses. $CUL3^{BPM}$ targets HB6 for ubiquitylation and degradation, affecting some ABA responses, such as stomatal behaviour and leaf serration[26]. Additional targets of BPMs in ABA signalling are PROTEIN PHOSPHATASE 2Cs (PP2Cs), which are negative regulators in the ABA pathway. BPM3 and BPM5 interact with multiple PP2Cs in the nucleus and promote their ubiquitylation and turnover in an ABA-dependent manner[27]. Some reports also revealed that other BTB/POZ proteins regulate ABA signalling. The BTB–ARM protein ARIA interacts with the transcription factor ABSCISIC ACID RESPONSIVE ELEMENT BINDING FACTOR 2, which controls ABA-dependent gene expression. In additon, genetic analysis showed that ARIA is involved in the ABA response. Two additional *Arabidopsis* BTB/POZ proteins, ABA HYPERSENSITIVE BTB/POZ PROTEIN 1 (AHT1) and BTB/POZ PROTEIN HYPERSENSITIVE TO ABA 1 (BPH1), were shown to negatively regulate ABA-mediated cellular events through genetic analysis. Although lacking evidence for their roles as subunits of CRL3s, these reports suggest that different types of BTB/POZ proteins regulate ABA signalling[58,62,63].

Recent findings revealed that $CUL3^{BPM}$ also contributes to jasmonate signalling. $CUL3^{BPM}$ targets MYC2/3/4, key transcriptional regulators of jasmonate response, for degradation. Interestingly, the stability of BPM3 is enhanced by jasmonate, indicating a negative feedback regulatory loop to reduce MYC levels and activities[28]. Of note, BPMs also regulate abiotic stress. BPMs interact with members of ETHYLENE-RESPONSIVE ELEMENT-BINDING FACTOR/APETALA 2 (ERF/AP2) transcription factors in *Arabidopsis*, which regulate stress responses[29]. Recently, the ERF/AP2 protein RELATED TO AP2.4 (RAP2.4) in *B. rapa* has been shown to assemble with AtBPM3 and AtCUL3, indicating that $CUL3^{BPM}$ functions in BrRAP2.4 degradation and abiotic stress response[30]. Adding more evidence to this, BPMs also interact with DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 2A (DREB2A), which is a key transcription factor controlling plant response to drought and heat stress. BPM2 constitutively localizes to the nucleus and interacts with DREB2A, while BPM4 translocates from the cytoplasm to the nucleus upon heat stress to assemble with DREB2A. By mediating DREB2A degradation, BPMs negatively modulate the heat stress response to avoid the effects on plant growth of excess DREB2A[31]. *A. thaliana* STRESS-INDUCED BTB PROTEIN1 (SIBP1), a BTB domain-containing protein, is involved in tolerance to salt stress and functions as a potential substrate adaptor for a CRL3 (ref.[64]). Besides the hormones noted above, salicylic acid-triggered plant immunity is also regulated by CRL3s, which we introduce later.

Light signalling.

CRL3s have also been implicated in light signalling. NPH3, a BTB–NPH3 family member in *Arabidopsis*, functions as a CRL3 substrate adaptor and regulates the ubiquitylation of phototropin1 (phot1) in response to different blue light intensities[65]. Under high-intensity blue light, $CUL3^{NPH3}$ mediates polyubiquitylation of phot1, which is targeted for proteasomal degradation. In contrast, under low-intensity blue light, phot1 seems to be exclusively mono-/multiubiquitylated, probably leading to the clathrin-associated endocytosis of the protein from the plasma membrane[65]. The dephosphorylation of NPH3 stimulated by blue light may also be crucial for phot1-dependent phototropism[66]. ROOT PHOTOTROPISM 2 (RPT2) is another BTB–NPH3 protein that contributes to phototropic response and regulates phot1 (refs.[67–70]). Whether RPT2 assembles with CUL3 and functions as a CRL3 remains to be resolved.

In addition to blue light, CRL3s also play roles in red light response and related biological processes. The substrate adaptors LRB1/2 control the protein stability of phytochrome B/D (phyB/D) and PHYTOCHROME INTERACTING FACTOR 3 (PIF3), a transcription factor that binds to phyB[51]. These studies indicate that light-activated phyB induces phosphorylation of PIF3 through a direct interaction, and that this phosphorylation enhances the affinity of PIF3 for the LRBs, resulting in degradation of both PIF3 and phyB[53]. Degradation of PIF3 alters the transcription of downstream target genes, while phyB degradation is a feedback regulatory event that desensitizes cells to red light[53]. The abundance of another PIF family member, PIF4, is also regulated by a CRL3. In this case, $CUL3^{BOP1/2}$ mediates the ubiquitylation of PIF4, thus promoting photomorphogenesis and thermomorphogenesis[47]. Interestingly, phosphorylation of PIF4 may not be required for BOP2 to bind PIF4, which is different from PIF3 and $CUL3^{LRBs}$ (ref.[47]).

Plant immunity.

Being sessile organisms, plants have evolved a number of mechanisms to defend themselves against microbial pathogens, and some of these involve CRL3s. A series of exciting studies conducted over the past 20 years revealed that a class of BTB/POZ proteins called NPR1/3/4 play integral roles in salicylic acid-induced immune signalling. Salicylic acid regulates systemic acquired resistance (SAR) as well as elicitor-triggered immunity (ETI) and associated programmed cell death (PCD). NPR1, a member of the BTB–ankyrin family, has a complex role in immunity since it promotes SAR but inhibits ETI[71]. The protein was originally characterized as a transcriptional co-activator that is required for SAR[72]. When salicylic acid levels are low, NPR1 forms high-molecular-weight oligomers in the cytoplasm. Salicylic acid promotes NPR1 monomer formation by altering the cellular redox state, leading to a reduction of key cysteine residues that contribute to oligomerization. Once released, the monomers are translocated to the nucleus where they promote the transcription of genes involved in SAR[72–74]. In a very recent study, salicylic acid was also shown to promote the formation of NPR1 condensates called salicylic acid-induced NPR1 condensates (SINCs) in the cytoplasm[75]. The NPR1 in SINCs assembles into $CUL3^{NPR1}$ E3 ligases, which mediate the degradation of proteins such as ENHANCED DISEASE SUSCEPTIBILIY 1 (EDS1) and WRKY54/70, promoting cell survival[75].

Degradation of NPR1 is also an important aspect of the immune response. NPR3 and NPR4—homologues of NPR1—assemble into E3 ligases and bind to NPR1 in a salicylic acid-dependent manner, resulting in its degradation. NPR3 and NPR4 both bind salicylic acid but with different affinities and outcomes[74,76]. Salicylic acid binds NPR3 with low affinity and promotes NPR1 binding and degradation. In contrast, the hormone binds NPR4 with high affinity but inhibits NPR1 binding. Interestingly, NPR1 displays minimal salicylic acid-binding activity despite a high level of conservation in the salicylic acid-binding core. A recent structural study showed that the loss of salicylic acid binding is due to changes in two key residues within this region of these proteins[77]. So far, the significance of the complex behaviours of the three NPR proteins to the immune response is not clear. However, it has been proposed that, in the absence of a pathogen when salicylic acid levels are low, $CUL3^{NPR4}$ promotes the degradation of most but not all of the nuclear NPR1 to prevent spurious activation of resistance[71]. When a pathogen infects a cell, salicylic acid levels increase, thus inhibiting $CUL3^{NPR4}$ but promoting $CUL3^{NPR3}$-mediated degradation of NPR1. Reduced NPR1 levels permit ETI and PCD of the infected cell. In neighbouring cells, the situation is different. Salicylic acid levels are presumably lower than in the infected cell and NPR3 no longer binds NPR1, thus enabling the accumulation of NPR1 and establishment of SAR[76]. Interestingly, CUL3a from rice was revealed to degrade rice NPR1 and negatively regulate cell death and immunity, suggesting that aspects of this pathway are conserved in rice[14].

NPR3 and NPR4 also facilitate degradation of the jasmonate transcriptional repressors JASMONATE ZIM-DOMAIN (JAZ) proteins and function in the early stages of ETI[78]. Jasmonate synthesis and signalling were shown to positively regulate ETI induction. During ETI, NPR3 and NPR4 bind to JAZ proteins—an interaction that is enhanced by salicylic

acid. NPR3/4 promote the degradation of JAZs, further activating jasmonate signalling and triggering de novo jasmonate synthesis[78].

Recently, NPR3 and NPR4 were shown to negatively regulate the stability of EDS1, a positive regulator of plant immunity[79]. Interestingly, PBS3, another important positive regulator of ETI, stabilizes EDS1 by reducing the interaction between EDS1 and NPR3/4, revealing a new mechanism by which plants positively regulate defence responses[79]. Similarly, Ca^{2+} negatively regulates salicylic acid-mediated plant defence through transcriptional repression of EDS1 via the CaM-binding transcription factor SIGNAL RESPONSIVE 1 (SR1)[80]. SR1-INTERACTION-PROTEIN 1 (SR1IP1), a BTB/POZ–NPH3 family member, functions as a substrate adaptor and targets SR1 for ubiquitylation and degradation, revealing how the negative regulation by Ca^{2+}/CaM/AtSR1 is relieved by $CUL3^{SR1IP1}$ to achieve effective plant defence response[81]. However, there are other CRL3s that negatively regulate plant immunity. $CUL3^{POB1}$ functions to mediate proteasomal degradation of U-BOX DOMAIN-CONTAINING PROTEIN 17 (PUB17), a positive regulator of plant immunity, thus suppressing hypersensitive response PCD[82]. *Nicotiana tabacum* POZ/BTB-CONTAINING PROTEIN (POB1), a homologue of *Arabidopsis* LRB2/POB1 in tobacco, functions as a substrate adaptor to recruit PUB17 in this process.

Of course, while plants evolved an immune system to protect themselves from infection, plant pathogens evolved effector proteins that suppress host immunity and promote pathogen growth. The *Phytophthora infestans* RXLR effector Pi02860 suppresses cell death mediated by the *P. infestans* elicitor INF1, causing leaf colonization and late blight disease of potato. *Solanum tuberosum* NRL1, a CRL3 substrate adaptor, interacts with Pi02860 and is required for its function, and hence acts as a susceptibility factor that negatively regulates plant immunity[83]. Furthermore, SWITCH-ASSOCIATED PROTEIN 70 (SWAP70), a guanine nucleotide exchange factor that positively regulates plant immunity, was identified as a candidate substrate of NRL1. NRL1 mediates the proteasomal degradation of SWAP70 and Pi02860 promotes this process by enhancing the interaction between NRL1 and SWAP70 (ref.[84]). Collectively, pathogens utilize $CUL3^{NRL}$ to negatively regulate plant immunity and promote disease.

Metabolic processes.

CRL3s also regulate metabolic processes in plants. BPMs interact widely with ERF/AP2 transcription factors. WRINKLED1 (WRI1), an ERF/AP2 that has a key role in fatty acid metabolism, is a target of $CUL3^{BPM}$. *bpm* knockdown lines exhibit bigger seeds and altered fatty acid content, confirming that BPMs participate in fatty acid metabolism through the regulation of WRI1 (ref.[32]). In apple, two BTB/TAZ proteins, BT1 and BT2, modulate degradation of the HLH104 transcription factor and negatively control iron uptake in response to a surplus of iron in plants[56]. Recently, apple BT2 was also reported to interact with MYB1, which is a key regulator in anthocyanin biosynthesis[85]. However, although MYB1 is degraded via the 26S proteasome pathway in this species, and BT2 promotes MYB1 ubiquitylation and degradation, CUL3A does not promote the degradation of MYB1. In addition, a compatible pull-down assay found that CUL3A represses the interaction

between BT2 and MYB1. Thus in apple, BT2 may regulate MYB1 and subsequent anthocyanin accumulation through a MdCUL3-independent pathway[85].

Regulation of CRL3s

Given the indispensable role of CRL3s in plant development, it is not surprising that the assembly, abundance and activity of the CRL3s are regulated at multiple levels. Some regulatory processes affect all CRLs. For example, dynamic modification of the cullin subunit by NEDD8/RUB, a small ubiquitin-like protein, is required for CRL activation and substrate adaptor exchange[86]. NEDD8/RUB conjugation to the cullin requires dedicated activation and conjugation enzymes while de-conjugation is accomplished by a multisubunit complex called the constitutive photomorphogenesis 9 (COP9) signalosome[87]. Other regulators of all CRLs include the CULLIN-ASSOCIATED AND NEDDYLATION-DISSOCIATED PROTEIN 1 (which functions in the CRL cycle) and the ABERRANT LATERAL ROOT FORMATION 4 protein (which functions as a negative regulator of CRLs)[88–93]. Here, we will focus on the regulation of specific CRL3s.

Control of subcellular partitioning of CRL3 components.

The activity of some CRL3s is regulated by localizing substrate adaptors and their substrates to different cellular compartments. As described earlier, NPR1, a key regulator of SAR, predominantly presents as an oligomer in the cytoplasm under normal conditions. Upon pathogen infection, NPR1 monomer is released and accumulates in the nucleus, where it is targeted by $CUL3^{NPR3/4}$ for subsequent ubiquitylation and degradation[76]. In another example, BPM4, a substrate adapter of DREB2A, translocates from the cytoplasm to the nucleus upon heat stress, where it interacts with DREB2A, indicating that $CUL3^{BPM4}$ ubiquitylates DREB2A upon stress conditions (Fig. 3a)[31]. In light signalling, the inactive forms of phyB and phyD are mostly localized to the cytoplasm. Photoactivation triggers their conversion to the active forms, along with rapid translocation into the nucleus, where they initiate downstream signalling and photomorphogenic responses. At the same time, phyB/D abundance is regulated by both $CUL4^{COP1}$ and $CUL3^{LRB1/2}$ (refs.[51,53,94]). In most cases, the mechanisms that regulate localization of substrate adapters or substrates are unknown.

Regulation of substrate recognition.

For most CRLs, substrate recognition occurs via a well-defined degron motif within the substrate. It is noteworthy that some CRL3 substrate adapters, such as BPMs, NPR3/4 and LRB1/2, interact with multiple substrates that share little or no structural similarities[24–32,51–53,76–79]. For example, BPMs recognize a number of divergent substrates (ERF/AP2s, PP2Cs, MYCs, AtHB6 and MYB56) involved in a variety of biological processes. Like its human orthologue, SPECKLE-TYPE POZ PROTEIN (SPOP), the SPOP-binding consensus (SBC; ϕ-π-S-S/T-S/T) or SBC-like motif (ϕ-π-S-*X*-S/T), where ϕ = nonpolar, π = polar and *X* = any amino acid, was shown to be important for the interaction of DREB2A with BPM2 in *Arabidopsis*. Further analysis showed that the majority of the proteins reported to interact with BPMs contain an SBC or SBC-like motif, suggesting the importance of the SBC motif in substrates for BPM recognition[31]. Notably, the SBC motif

is not highly conserved, allowing for recognition of a variety of substrate proteins. Studies of LRB–FRI and NPR–EDS1 recognition also characterized the regions involved in their respective interaction domains but did not identify conserved motifs[52,79].

CRLs are crucial regulators in plant hormone responses, and in some cases plant hormones are also essential for the regulation of substrate recognition in CRLs. In SCFs, auxin and jasmonate act as molecular glue for the formation of co-receptors[95,96]. In CRL3s, salicylic acid was also reported to affect NPR1–NPR3 and NPR1–NPR4 interactions (Fig. 3b). Salicylic acid promotes the NPR1–NPR3 interaction while disrupting the interaction between NPR1 and NPR4, leading to different effects on plant immunity[76,77]. In the case of NPR4, a recent structural study showed that salicylic acid binding to the salicylic acid-binding core results in a conformational change that disrupts the NPR1–NPR4 interaction[77]. Salicylic acid is also necessary for some NPR3/4–JAZ interactions in jasmonate signalling[78]. For BPM–PP2C interactions in ABA signalling, although ABA is not necessary for these interactions, it enhances the degradation of PP2C[27].

Regulation of substrate adaptor stability.

Another level of CRL regulation is through modulating the stability of substrate adapters. In the absence of substrates, substrate adapter ubiquitylation is prevalent in cullin-based E3 ligases in non-plant systems[97]. A similar process has been proposed in plants[98]. This auto-ubiquitylation may result in degradation of substrate adapters, enhancing the availability of cullin/RBX1 to assemble into new CRLs with different substrate adapters. Many CRL3 substrate adapters were found to be short lived and accumulated after proteasome inhibitor MG132 treatment, implying that they are targets of the UPS. In plants, such examples include BPMs and LRB1/2 (refs.[26,51]). The protein stability of BPM1 has been shown to be downregulated by darkness and salt stress and upregulated by high temperature, suggesting its potential role in these biological processes[25]. Substrate adapters may also be destabilized by other proteins, resulting in stabilization of the respective target substrates. For example, it was reported that 14-3-3 proteins interact with and de-stabilize $CUL3^{ETO1/EOL}$ E3 ligases. Surprisingly, 14-3-3 proteins also interact with the substrates of these E3s, type 2 ACSs, but in this case they act to stabilize the ACS. Thus 14-3-3 proteins act to increase the levels of ACS proteins though a direct interaction and by decreasing the stability of $CUL3^{ETO1/EOL}$ (ref.[99]).

Substrate adaptors form dimers to improve ubiquitylation.

Many substrate adaptors of CRLs dimerize to generate dimeric ubiquitin E3 ligases. Multiple examples have been well investigated in yeast and humans, such as the dimerization of Cdc4, Fbw7, Pop1/2, Keap1 and SPOP[19,20,100–105]. According to these studies, dimerization of CRLs improves ubiquitylation by two mechanisms. In the first mechanism, two substrate-binding sites and two E2 catalytic sites in one structure may allow the accommodation of different-sized substrates, and increase the efficiency of ubiquitin chain initiation and elongation[100,102,103,106]. In the second mechanism, for substrates with two or more degrons, dimerization of E3s enhances the substrates avidity through providing multiple substrate-binding domains[20,104,107]. Although not as well studied, many BTB/POZ proteins in plants form homodimers or heterodimers with family members

through BTB/POZ domains. For example, NPH3 and RPT2 heterodimerize through BTB domains; NPR3 and NPR4 as well as LRB1 and LRB2 associate with themselves and each other; and BPMs were also reported to form homodimers and heterodimers[26,51,70,76,82]. The BPM orthologue SPOP in humans is essential for many cellular processes[108–110]. SPOP dimerizes through its BTB domain and interacts with CUL3 (ref.[20]). Structural analysis showed that in addition to the MATH and BTB domains, there is an additional domain near the C terminus called the BACK domain. This domain further promotes SPOP assembly into oligomers and greatly enhances ubiquitylation activity[19,104]. Moreover, SPOP localizes in nuclear speckles with liquid droplet properties, and defective oligomerization of SPOP disrupts nuclear speckle localization and reduces the efficiency of ubiquitylation[111]. These results suggest a model in which oligomer formation promotes efficient ubiquitylation of various substrate proteins. Interestingly, BPMs also form nuclear speckles, raising the possibility that BPMs are regulated by oligomer formation[26,27].

Crosstalk between CRL3 and other E3 ligases.

Many important cellular processes, signalling pathways and proteins need to be tightly regulated via multiple mechanisms. In recent years, the characterization of gene regulatory networks has illustrated the complexity of transcriptional regulation[112]. Now, a similar picture is emerging in studies of E3 ligases. In some cases, different E3s control the same pathway or even the same substrate protein. Other E3s, such as $CUL3^{BPM}$, regulate multiple hormone-related pathways and may have an integrative function. For example, in the ABA signalling pathway, ABA co-receptors (PP2Cs) are negative regulators of the ABA response. Recent studies have shown that $CUL3^{BPM}$ promotes their degradation in an ABA-dependent manner[27]. Meanwhile, the U-box E3 ligases PUB12 and PUB13 interact with ABA-INSENSITIVE 1 (a PP2C protein) and mediate its degradation[113]. Furthermore, PP2CA levels are regulated by RGLG1/5 RING-type E3 ubiquitin ligases[114,115]. At this point, it is not clear why the same group of proteins are targeted by diverse E3s, but presumably this mechanism facilitates the integration of information from multiple sources including the environment. In another example involving hormones, the E3 ubiquitin ligase PUB10 targets MYC2 for proteasomal degradation during jasmonate responses[116] while $CUL3^{BPM}$ also regulates MYC2/3/4 stability and function in jasmonate signalling[28]. DREB2A regulates heat and drought responses and is degraded by two C3HC4 RING–domain-containing proteins, DREB2A-INTERACTING PROTEIN 1/2 (ref.[117]). However, DREB2A protein levels were still low in *drip1 drip2* under heat stress conditions, indicating that other E3 ligases are responsible for DREB2A degradation[118]. As described above, $CUL3^{BPM}$ was also found to mediate DREB2A degradation[31]. In another example, the ubiquitylation and degradation of phyB photoreceptors is mediated by CUL4–COP1 E3 ligase in a PIF-promoted manner, and $CUL3^{LRB1/2}$ regulates the degradation of both PIF3 and phyB in response to red light[51,53,94]. Recently, $SCF^{EBF1/2}$ was also shown to be involved in light-induced PIF3 degradation[119]. Compared with $CUL3^{LBRs}$, EIN3-BINDING F BOX PROTEIN 1/2 only targets PIF3 for ubiquitylation and degradation without affecting the stability of phyB. $CUL3^{LRB}$ plays predominant roles under strong red light, while $SCF^{EBF1/2}$ functions in response to a wide range of light intensities. In a final example, the LFY transcription factor has previously been shown to be regulated by SCF^{UFO}, and

recent findings showed that $CUL3^{BOP2}$ also regulates the degradation and activity of LFY to promote flower development[48,60].

Conclusion

Recent studies have clearly illustrated the importance of CUL3 E3 ligases to plant growth and development. Nevertheless, there is still much to learn about these ligases, including uncharacterized functions of the CRL3s, the evolutionary history of CRL3 components and the precise regulation of CRL3 activities. With the development of novel high-throughput approaches, we anticipate that more CRL3 substrates will be identified. New powerful techniques such as CRISPR–Cas9 also help to analyse their functions by quickly generating high-order mutants of BTB/POZ gene families. In addition, as we begin to appreciate the complex roles of E3s in diverse aspects of growth and development, it will be important to apply network-based approaches to their study.

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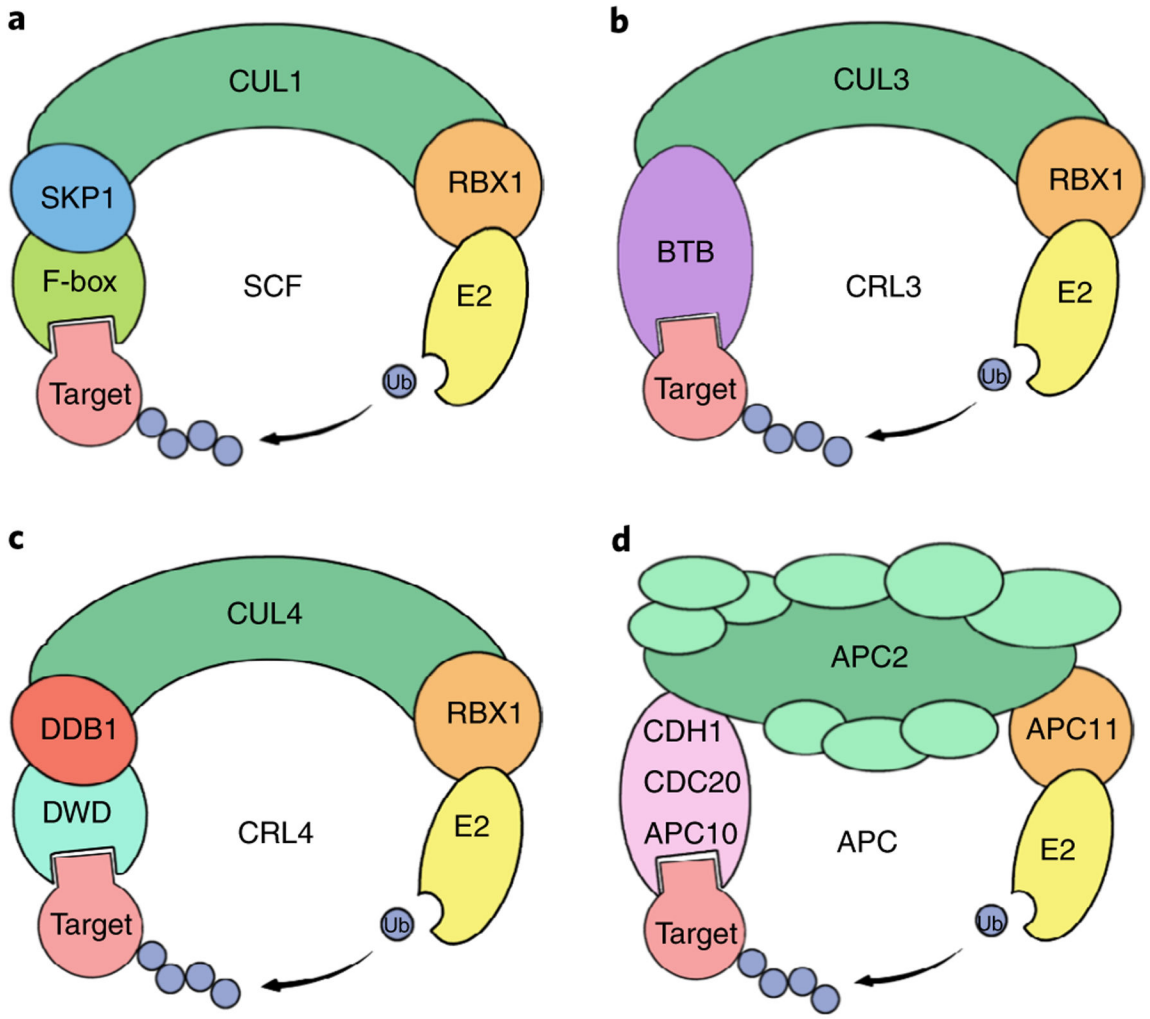

Fig. 1 |. Subunit composition of CRLs in plants.

a, CUL1 E3 ligases utilize SKP1 to bind CUL1 and F-box proteins to target substrates. **b**, In CUL3 E3 ligases, BTB/POZ proteins interact with both CUL3 and substrates. **c** E3 ligases use DAMAGE-SPECIFIC DNA-BINDING PROTEIN 1 (DDB1) to bind CUL4 and WD40 domain-containing DWD proteins for target recognition[120]. **d**, The APC contains 11 or more subunits. APC2 and APC11 function is similar to cullins and RBX1, respectively, while CDC20, CDH1 and APC10 function as substrate adaptors[121].

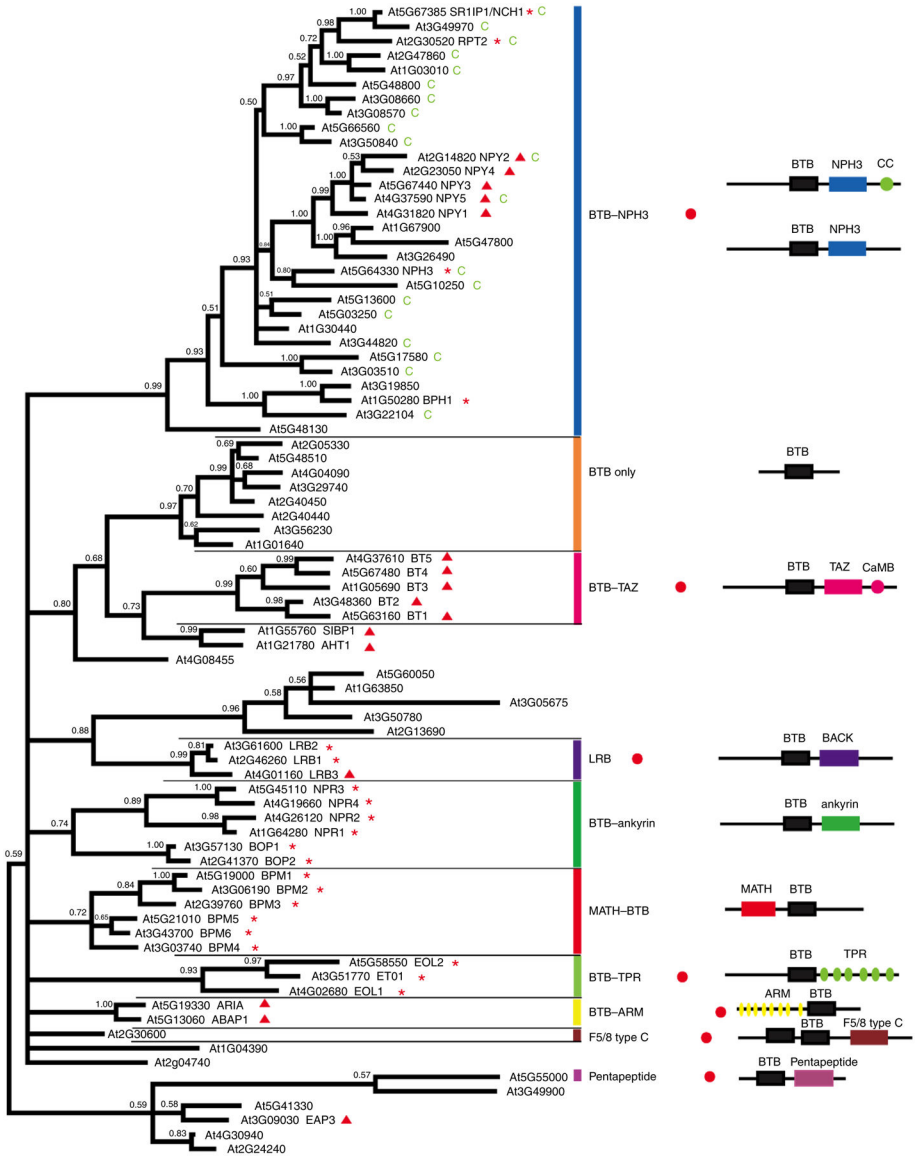

Fig. 2 |. Phylogenetic analysis of the BtB/POZ protein family from *Arabidopsis*.

A total of 80 BTB/POZ sequences from *Arabidopsis* were aligned using Clustal W and their BTB domains were used to generate the tree. Phylogenetic trees were inferred using MrBayes version 3.2.7 (ref.[122]). Two runs of four chains were run for 15 million generations using the parameters aamodelpr = mixed, nst = 6 and rates = invgamma. Posterior probabilities of >0.5 are shown for corresponding nodes. The subfamilies identified from the phylogenetic analysis and their corresponding domains are marked on the right. BTB/POZ proteins that have been functionally identified as substrate adaptors of CRL3s are marked with red asterisks. Those that have been studied but not experimentally shown to bind CUL3 are marked with red triangles. red circles indicate subfamilies that only exist in plants. The BTB–nPH3 proteins that have an additional coiled-coil domain in their structures are marked with a green letter ‘C’.

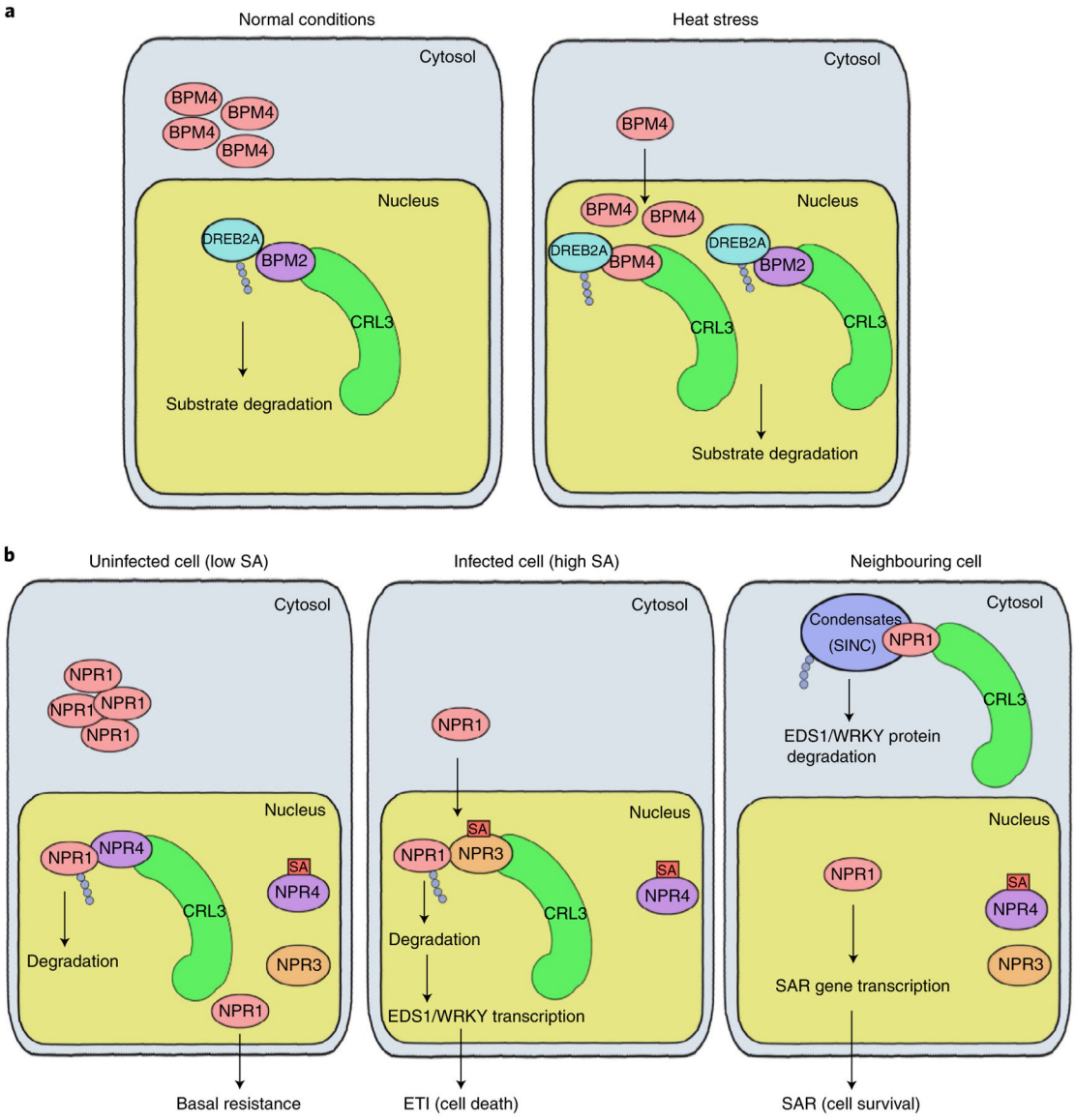

Fig. 3 |. Examples for CRL3 regulation.

a, Control of subcellular partitioning of CRL3 components. While BPM2 constitutively localizes to the nucleus and mediates DREB2A ubiquitylation, BPM4 translocates from the cytoplasm to the nucleus upon heat stress, where it interacts with DREB2A and promotes its degradation. By mediating DREB2A degradation, BPMs negatively modulate the heat stress response to avoid the effects on plant growth of excess DREB2A[31]. **b**, Salicylic acid (SA) regulates $CUL3^{NPR1/3/4}$ substrate function. NPR1 is a master regulator of SAR in salicylic acid-regulated plant immunity. In an uninfected cell, basal levels of salicylic acid lead to $CUL3^{NPR4}$-mediated degradation of most of the NPR1 to prevent spurious activation of resistance. Pathogen infection increases salicylic acid levels, which inhibits $CUL3^{NPR4}$ but promotes $CUL3^{NPR3}$-mediated degradation of NPR1 to allow EDS1 and WRKY transcription and establish ETI. In neighbouring cells, where salicylic acid levels are lower, $CUL3^{NPR3}$-mediated degradation of NPR1 is reduced, enabling the accumulation

of NPR1 and transcription of SAR genes; in the cytoplasm, salicylic acid also induces the formation of NPR1 condensates (SINCs), which mediate $CUL3^{NPR1}$ degradation of cytoplasmic proteins, such as EDS1 and WRKY transcription factors[75–77].

Table 1 |

Functions of various CRL3s in different biological processes in plants

Adaptor name	Protein subfamily	Substrate	CRL3 E3 function	Physiological function	Reference(s)
ETO1/EOL1/ EOL2	BTB-TPR	Type 2 ACSs	ETO1 directly inhibits ACS5 enzyme activity and interacts with CUL3 to mediate ACS5 degradation. EOL1 and EOL2 also contribute to ACS degradation.	Negatively regulates ethylene production.	49,50,61
BPMs	MATH-BTB	HB6	$CUL3^{BPM}$ targets AtHB6 for ubiquitylation and degradation.	Promotes ABA response.	26
	MATH-BTB	PP2Cs	$CUL3^{BPM3/5}$ targets PP2Cs for degradation in the nucleus.	Positively regulates ABA signalling.	27
	MATH-BTB	MYC2/MYC3/ MYC4	$CUL3^{BPM}$ promotes degradation of MYC2/MYC3/MYC4.	Resets signalling and prevents harmful runaway jasmonate responses.	28
	MATH-BTB	ERF/AP2s, RAP2.4	$CUL3^{BPM}$ promotes ERF/AP2 degradation.	Regulates stress responses.	29,30
	MATH-BTB	DREB2A	$CUL3^{BPM2/4}$ promotes DREB2A degradation through the negative regulatory domain.	Negatively regulates heat stress response and prevents adverse effects of excess DREB2A.	31
	MATH-BTB	WRI1	$CUL3^{BPM}$ promotes degradation of WRI1.	Regulates fatty acid contents in seeds.	32
	MATH-BTB	MYB56	$CUL3^{BPM}$ promotes degradation of MYB56.	Positively affects flowering.	24,25
ZmMAB1	MATH-BTB	p60 of katanin	ZmMAB1 interacts with CUL3a. p60 is a candidate substrate.	Necessary for normal meiosis, mitosis and germline development.	33
TaMAB2	MATH-BTB	eIF3 and eIF4	TaMAB2 directly interacts with CUL3 and eIF3 and eIF4.	Regulates translation initiation during the onset of embryogenesis.	37
NPR3/NPR4	BTB-ankyrin	NPR1	NPR3 and NPR4 function as CRL3 adaptors to mediate NPR1 degradation in a salicylic acid-regulated manner in the nucleus.	Regulates SAR and ETI.	76,77
NPR3/NPR4	BTB-ankyrin	JAZ1	Salicylic acid enhances the interaction between NPR3/NPR4 and JAZs and promotes the degradation of JAZ1 at the early stage of ETI.	Mediates activation of early jasmonate signalling and synthesis, which is essential for RPS2-mediated ETI.	78
NPR3/NPR4	BTB-ankyrin	EDS1	NPR3 and NPR4 promote the degradation of EDS1.	Negatively regulates plant immunity.	79
NPR1	BTB-ankyrin	EDS1 WRKY54/70	Salicylic acid induces NPR1 condensates (SINCs) in the cytoplasm, promoting the formation of $CUL3^{NPR1}$ and the degradation of EDS1 and WRKY54/70.	Promotes cell survival during ETI.	75
BOP2	BTB-ankyrin	LFY	BOP2 promotes ubiquitylation of LFY in vitro and also regulates LFY activity.	Promotes flower development.	48
BOP1/2	BTB-ankyrin	PIF4	BOPs promote ubiquitylation of PIF4 in vitro.	Promotes photomorphogenesis and modulates thermomorphogenesis.	47
NPH3	BTB-NPH3	phot1	NPH3 promotes the degradation of phot1.	Regulates the blue light response.	65
SR1IP1	BTB-NPH3	AtSR1	SR1IP1 promotes the degradation of AtSR1.	Positively regulates plant immunity.	81
StNRL1	BTB-NPH3	SWAP70	StNRL1 promotes the degradation of SWAP70 in concert with the *Phytophthora* effector protein Pi02860.	Negatively regulates plant immunity to promote disease.	83,84

Adaptor name	Protein subfamily	Substrate	CRL3 E3 function	Physiological function	Reference(s)
LRB1/2	LRBs	phyB/phyD	LRB1/2 controls the stability of phyB/phyD.	Negatively regulates photomorphogenesis.	51
LRB1/2	LRBs	PIF3/phyB	$CUL3^{LRB}$ mediates the degradation of both PIF3 and phyB.	Feedback regulation in response to red light.	53
LRB1/2	LRBs	FRI	LRB1/LRB2 promote FRI degradation.	Negatively modulates flowering in response to vernalization.	52
NtPOB1	LRBs	PUB17	POB1 interacts with PUB17 in the nucleus and promotes its degradation.	Suppresses hypersensitive response PCD in innate immune responses.	82
MdBT1/MdBT2	BTB-TAZ	MbHLH104	MdBT1/MdBT2 promote the degradation of MdbHLH104.	Maintains iron homeostasis under iron surplus conditions.	56