

# Ubiquitination-mediated protein degradation and modification: an emerging theme in plant-microbe interactions

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Post-translational modification is central to protein stability and to the modulation of protein activity. Various types of protein modification, such as phosphorylation, methylation, acetylation, myristoylation, glycosylation, and ubiquitination, have been reported. Among them, ubiquitination distinguishes itself from others in that most of the ubiquitinated proteins are targeted to the 26S proteasome for degradation. The ubiquitin/26S proteasome system constitutes the major protein degradation pathway in the cell. In recent years, the importance of the ubiquitination machinery in the control of numerous eukaryotic cellular functions has been increasingly appreciated. Increasing number of E3 ubiquitin ligases and their substrates, including a variety of essential cellular regulators have been identified. Studies in the past several years have revealed that the ubiquitination system is important for a broad range of plant developmental processes and responses to abiotic and biotic stresses. This review discusses recent advances in the functional analysis of ubiquitination-associated proteins from plants and pathogens that play important roles in plant-microbe interactions.

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## Introduction

Plants are routinely exploited by a spectrum of parasites, such as viruses, bacteria, fungi, nematodes, and insects in their life cycles. Unlike animals, plants are sessile and lack a somatically adaptive immune system in responding to pathogen infections and insect attacks. Instead, plants have evolved other multifaceted mechanisms to protect themselves from various biotic and abiotic challenges [1]. Both pre-formed defense barriers and induced defense responses are employed by plants to counteract potential pathogen invasion [2]. Constitutive barriers, such as cuticular wax layers, pre-formed antimicrobial enzymes, and secondary metabolites, could function as the first fortification that pathogens must penetrate or bypass. Induced

defense reactions are mounted when the pathogen attacks are perceived by the hosts. Such pathogen recognition and subsequent induction of defense responses is critical to the protection of plants if preformed barriers are overcome by an evolutionarily active population of pathogens, which is frequently the case in nature. Based on whether the attacked plants are natural hosts of the pathogen or not, resistance to pathogens can be divided at 'species' and 'cultivar' levels, respectively [3]. The species-level resistance, also known as non-host resistance, refers to the fact that any given pathogen can only cause disease on a limited range of plant species. The cultivar-level resistance is also called race-specific resistance. During the process of co-evolution with the pathogens, plants have evolved resistance (*R*) genes to specifically recognize cognate avirulence (*Avr*) genes from the pathogens [4]. Such race-specific plant-pathogen interactions conform to the gene-for-gene model, in which the presence of a plant R protein and its corresponding pathogen-encoded *Avr* gene product leads to resistance, while lack of functional products of either gene results in disease [5]. Recognition of the *Avr* gene product

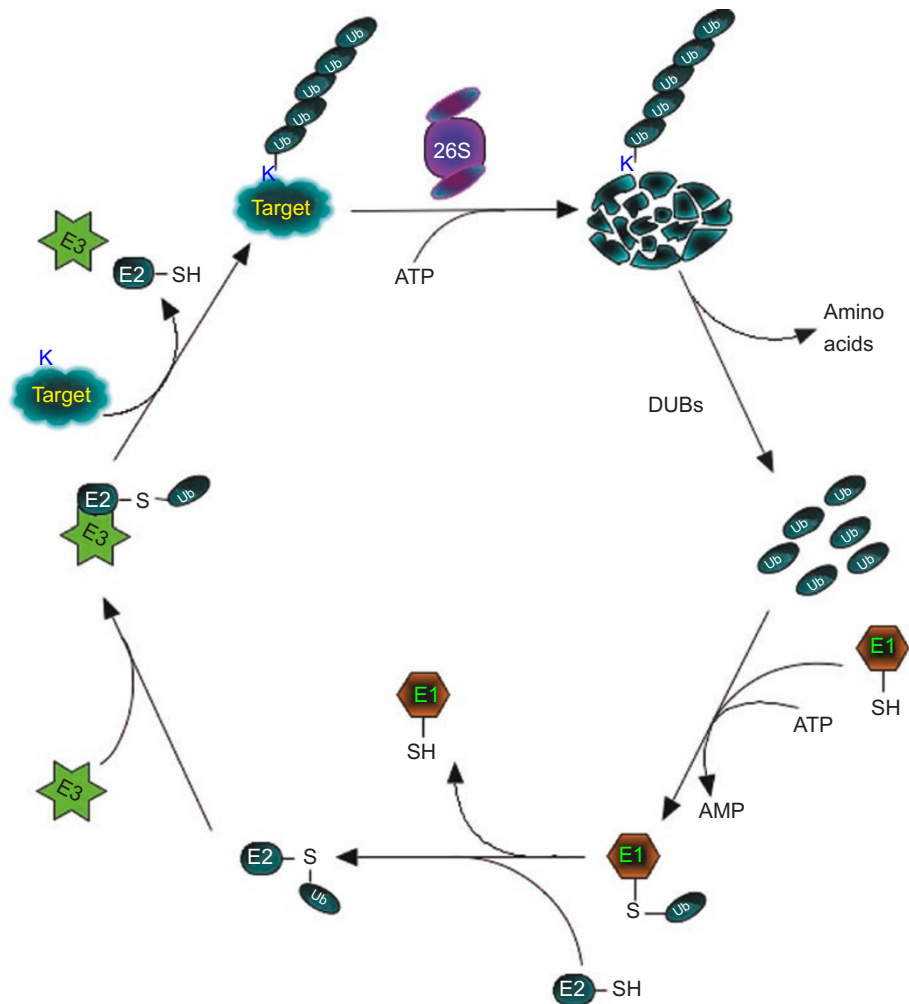
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**Figure 1** The Ubiquitin/26S proteasome pathway. The protein ubiquitination begins with the activation of the ubiquitin molecule (Ub) in an ATP-dependent manner. The activated ubiquitin is then transferred to the active site of the ubiquitin-conjugating enzyme (E2). Finally, an ubiquitin-ligase (E3) binds E2 and catalyzes the formation of an isopeptide linkage between the activated ubiquitin and the Lysine residue of the substrate protein. After a chain of multiple ubiquitins is attached to the target protein, it is usually destined to the 26S proteasome, where the target protein is degraded and the ubiquitin monomers are reclaimed by the action of de-ubiquitination enzymes (DUBs).

by the R protein triggers a complex of signal transduction cascade culminating at local (e.g. hypersensitive response (HR)) and global (e.g. systemic acquired resistance (SAR)) defense mobilization in the plant [6, 7]. Over the years, the gene-for-gene model has been refined. In the “guard” hypothesis, R proteins only ‘recognize’ the Avr protein indirectly, for example after it complexes with other host protein(s), such as RIN4 in *Arabidopsis* [8, 9]. These host proteins are regarded as virulence targets. Through the binding of the pathogen Avr protein (also termed as effector) to the target proteins, a susceptible reaction ensues. In the case of resistance, it has been proposed that the R gene

product evolved to recognize the Avr-dependent binding or modification of the host target protein (s) [10]. To date, over 50 R genes have been isolated from different plant species, and key components in the defense signal transduction pathways in R-gene-mediated resistance, such as *enhanced disease susceptibility (EDS1)* and *required for MLA resistance1 (RAR1)*, have also been identified [11, 12]. Extensive reviews of R genes and R-gene-mediated defense signaling pathways are available elsewhere [13]. Here, we will focus our discussion on recent findings regarding the involvement of the ubiquitination machinery in plant-microbe interactions and plant defense signaling.

## The ubiquitin/26S proteasome system

Two distinct yet coordinate steps are involved in the degradation of a protein by the ubiquitin system: (i) covalently attaching multiple ubiquitin molecules to the target protein; (ii) degrading the modified protein by the 26S proteasome (Figure 1). Ubiquitination of the target protein is achieved by three sequential enzymatic reactions that act in concert. Initially, the ubiquitin-activating enzyme, E1, catalyzes the activation of the ubiquitin molecule, an evolutionarily conserved protein of 76 residues, in its C-terminal Gly to a high-energy thiol ester intermediate. Following activation, an ubiquitin-conjugating enzyme, E2 (also called ubiquitin-carrier proteins, UBC), catalyzes the transfer of the activated ubiquitin moiety from E1 to a member of the ubiquitin-protein ligase (E3) family. E3 catalyzes the last step of the conjugation process, transferring the ubiquitin from the E2 to a lysine residue in the target protein, often by forming an intermediate complex with the E2 and the protein substrate. After the first ubiquitin moiety is transferred to the protein target, a polyubiquitin chain is then synthesized by transferring additional activated ubiquitin moieties to Lys48 of the previously conjugated ubiquitin molecule. The attached polyubiquitin chain most likely will serve as a signal recognized by the 26S proteasome. In addition to the formation of the polyubiquitin chain through the Lys48, monomeric ubiquitylation and polyubiquitylation through the Lys29 or Lys63 residues of ubiquitin have also been reported and it is thought that these alternative ubiquitination events may be involved in cellular processes other than degradation [14].

The 26S proteasome is a giant self-compartmentalized protease that is composed of a 20S barrel-shaped core particle (CP) with an inner proteolytic chamber and two flanking 19S polar regulatory particles (RP), where protein recognition and ATP-dependent unfolding occur [15]. In eukaryotes, the 20S CP is composed of four stacked heptameric rings that are made of related  $\alpha$  and  $\beta$  subunits in an  $\alpha_{1-7}/\beta_{1-7}/\beta_{1-7}/\alpha_{1-7}$  configuration [16]. The two central  $\beta$ -subunit heptameric rings form the catalytic chamber. Three of the  $\beta$  subunits,  $\beta_1$ ,  $\beta_2$ , and  $\beta_5$ , are post-translationally processed at their amino terminus to yield an active protease site. The  $\alpha$ -subunit rings at the two outer sides of the central  $\beta$ -subunit rings form a gated channel that controls the passage of the unfolded substrates and cleaved peptides in and out of the proteolytic chamber. The 19S RP particle is made of two subcomplexes, the lid and the base. The base subcomplex contacts with the  $\alpha$ -subunit ring of the 20S CP and contains a ring of six AAA-ATPase subunits (RPT1-6) along with three non-ATPase subunits (RPN1, RPN2, and RPN10). Generally, the lid subcomplex encompasses eight stoichiometric subunits (RPN3, RPN5,

RPN6, RPN7, RPN8, RPN9, RPN11, and RPN12) [17]. However, some non-essential subunits may transiently bind to the lid subcomplex in regulating 26S proteasome activity and localization [18, 19]. In addition, the deubiquitinating enzymes (DUBs) that cleave the  $\epsilon$ -amide bond between ubiquitin and a lysine side chain to recycle the ubiquitin molecules from the substrate proteins are also found in the 19S subcomplex [16]. Unlike the 20S CP, of which the structure, assembly, and enzymatic mechanism have been elucidated, the organization and specific role of most subunits of the 19S complex remain unknown [16]. Several substrate-recruiting proteins, probably including chaperones, have been implicated in helping transfer the ubiquitinated protein from the ubiquitination machinery to the 26S proteasome [20].

## Diversity of plant E3 ligases

One important feature of the ubiquitination pathway is its hierarchical structure in terms of the number of E1s, E2s, and E3s in a given genome. For example, the *Arabidopsis* genome encodes 2 E1s, 41 E2s, and over 1200 E3 components [21, 22]. In the ubiquitin system, E3 ubiquitin ligase plays a central role in selecting the myriad of appropriate candidate proteins during the ubiquitination process [23]. This is manifested by the fact that E3s are the most diverse components in the ubiquitination pathway [15]. Until recently, four different families of ubiquitin ligases had been described based on their subunit composition and mechanism of action: HECT, SCF, APC, and RING/U-box [22]. The HECT (homologous to E6-AP COOH terminus) domain was initially identified in the viral E6-associated protein (E6-AP) during the study of human papilloma viruses [24, 25]. It is composed of ~350 amino acids and its C-terminus contains a conserved active cysteine residue required for the ligase function. The HECT-type ligase is the only known E3 that forms an E3-ubiquitin thiolester intermediate before the final attachment of ubiquitin to the substrate [24]. The SCF-type E3 ligase is typically composed of four different polypeptides: SKP1 that serves as an adaptor protein; Cullin1 (CDC53); RING-finger protein RBX1 (or ROC1 and HRT1) that interacts with the Cullin1 and E2-conjugating enzyme; and an F-box protein that is responsible for recruiting substrates. Structural analysis indicated that Cullin1 (most of the time abbreviated as CUL1) acts as a scaffold that directs other members of the SCF complex to bring the ubiquitin-loaded E2s and the substrate in proximity to promote the transferring of ubiquitin directly from the E2 to the substrates [26]. Besides Cullin1, recent data indicated that Cullin2 and Cullin3 also form similar groups of E3 ligases, in which an elongin C-SOCS module or a BTB protein replaces the SKP1-F-box protein module

**Table 1** Members of the ubiquitin/26S proteasome system implicated in plant defense against biotic and abiotic challenges

Gene	Protein type	Host	Challenge	Refs
<b>Biotic challenges</b>				
ACRE132	RING-H2 (E3)	Tobacco/tomato	<i>C. fulvum</i>	[120]
ACRE189	F-box (E3)	Tobacco/tomato	<i>C. fulvum</i>	[45]
ACRE276	U-box (E3)	Tobacco/tomato	<i>C. fulvum</i>	[45]
At2g35000	RING finger (E3)	<i>Arabidopsis</i>	<i>E. cichoracearum</i>	[44]
AtCMPG1/ AtCMPG2	U-box (E3)	<i>Arabidopsis</i>	Oligopeptide elicitor or <i>P. syringae</i>	[67]
ATL2/ATL6	RING-H2 (E3)	<i>Arabidopsis</i>	Elicitor chitin	[39, 40]
BRH1	RING-H2 (E3)	<i>Arabidopsis</i>	Elicitor chitin	[41]
COI1	F-box (E3)	<i>Arabidopsis</i>	Wounding, <i>P. mastophorum</i> , etc.	[47, 48, 121]
LeCOI1	F-box (E3)	Tomato	Spider mites	[52]
COP9	19S RP like	Tobacco	Tobacco mosaic virus	[61]
EL5	RING-H2 (E3)	Rice	N-acetylchitooligo-saccharide elicitor	[42]
NtE1A and 1B	E1	Tobacco	Tobacco Mosaic Virus, tomato mosaic virus, wounding	[43]
PcCMPG1 ( <i>ELI17</i> )	U-box (E3)	Parsley	Oligopeptide elicitor or <i>P. sojae</i>	[66]
RCE1	RUB-conjugating enzyme	<i>Arabidopsis</i>	-	[87]
SGT1	TPR domain (SCF E3 associated)	<i>Arabidopsis</i> or Barley or Tobacco	<i>P. parasitica</i> or powdery mildew or virus	[92-94]
SKP1	SCF subunit	tobacco	Tobacco mosaic virus	[61]
SON1	F-box (E3)	<i>Arabidopsis</i>	<i>P. parasitica</i> and <i>P. syringae</i>	[58]
SPL11	U-box (E3)	Rice	<i>M. grisea</i> and <i>X. oryzae</i> pv <i>oryzae</i>	[83]
<b>Abiotic challenges</b>				
AtCHIP	U-box (E3)	<i>Arabidopsis</i>	Temperature stress	[76]
BG55	U-box (E3)	Mangrove	High salinity	[122]
DaRub1	Polyubiquitin	<i>D. antarctica</i>	Cold stress	[123]
HOS1	RING-H2 (E3)	<i>Arabidopsis</i>	Cold stress	[124]
LeUBC1	E2	Tomato	CdCl <sub>2</sub> heavy metal stress	[125]
ORE9	F-box (E3)	<i>Arabidopsis</i>	Drought, oxidative stress	[126, 127]

in the Cullin1-type SCF E3 ligase, respectively [27-29]. In the *Arabidopsis* genome, around 700 genes are predicted to encode proteins containing the 40 amino-acid F-box motif [30]. The anaphase promoting complex (APC)-type E3s are complexes of over 10 proteins. The complex also contains a Cullin protein, although their action mechanism is distinct from that of SCF group E3s. It is expected that only a single or a few APC isoforms exist in plants [22, 31]. The RING (really interesting new gene) finger motif is characterized by the presence of Zn<sup>2+</sup>-chelating amino acid residues hallmarked as C3HC4 (C, cysteine; H, histidine) or C3H2C3, which forms two cross-brace-arranged free

loops. Like SCF E3s, the RING-type E3 ligases serve as scaffolds to bring together the activated ubiquitin-E2 and the substrate in promoting the transfer of ubiquitin to the substrate. In contrast to that only few HECT-type E3s are identified in plants [32], the SCF and the RING-type E3 are found in hundreds of proteins [33, 34]. The U-box is a novel E3 ubiquitin ligase activity-related protein domain that was first identified in the yeast ubiquitination factor UFD2 [35]. The U-box contains ~75 amino acids and possesses a tertiary structure resembling that of the RING finger [36, 37]. The major difference between the U-box and RING domains is that the U-box lacks the hallmark

Zn<sup>2+</sup>-chelating cysteine and histidine residues characteristic of RING fingers. Consequently, the conserved zinc-binding residues supporting the cross-brace arrangement in RING-finger domains are replaced by hydrogen-bonding networks in the U-box [37].

### Members of the ubiquitination system are implicated in plant defense signaling

By removing abnormal proteins, the ubiquitin/26S proteasome system performs an essential housekeeping role in the cell. Importantly, the system also serves as control/regulation mechanism by eliminating normal proteins, such as rate-limiting enzymes and key regulators, in different pathways. The first clue of the involvement of ubiquitination in plant defense came from the observation that the *Arabidopsis* R protein RPM1 is degraded coincidentally with the onset of the HR [38]. In the past several years, approximately a dozen ubiquitination-related components have been identified that are involved in plant-pathogen or plant-insect interactions (Table 1). These genes were identified from different plant species and most of them are induced after pathogen or elicitor challenges. For example, the RING-finger-type E3 ligases ATL2 and ATL6 were rapidly induced in *Arabidopsis* plants treated with chitin elicitor [39, 40]. Similarly, other members of RING-type E3 ligases such as EL5 from rice and BRH1 from *Arabidopsis* were also induced after elicitor treatment [41, 42]. The upregulation of these genes in plants suggested that they might be involved in the response to biotic stresses. However, whether they play a role in signaling or regulating plant defense remains to be determined. The induction of an E1 in tobacco plants after virus infection also supports the idea that ubiquitination is involved in plant-pathogen interactions [43]. Interestingly, the E1 enzymes NtE1A and NtE1B were induced in tobacco plants only when inoculated with tobacco mosaic virus (TMV) or tomato mosaic virus (ToMV), but not cucumber mosaic virus. In the examination of *Arabidopsis* global gene expression after treatment with chito-octamers and hydrolyzed chitin, Ramonell *et al.* [44] identified a RING-finger E3 ligase that showed early responsiveness to the elicitor treatment. Loss-of-function of the gene by T-DNA insertional mutation resulted in enhanced disease resistance when *Arabidopsis* plants were inoculated with low-density of *Erysiphe cichoracearum*, demonstrating that this E3 ligase is required for plant responses to powdery mildew invasion, probably in the initial signaling process to pathogen infection.

As mentioned above, E3 ubiquitin ligases are the most abundant members of the ubiquitination system and they are generally considered to be most important in controlling substrate specificity. Therefore, it is not surprising that

most of the components of the ubiquitination system that have been shown to play a role in the regulation of various pathways belong to the E3 group. Except for the HECT- and APC-type E3s, to which only a limited number of members have been identified in plants, all other classes of E3s have been implicated in plant defense. The *ACRE132* (*Avr9/Cf9* rapidly elicited), *ACRE189*, and *ACRE276* genes encode different types of E3 components. They were identified in a cDNA-AFLP analysis of Cf9 tobacco cell cultures treated with Avr9-containing intercellular fluid. Silencing of *ACRE189* and *ACRE276* dramatically reduced *Avr4/Cf4*- or *Avr9/Cf9*-induced HR, apparently suggesting that they are involved in the signaling of Cf4- and Cf9-mediated cell death and/or resistance [45]. However, further studies, such as phenotype of *ACRE* overexpression plants challenged with *Cladosporium fulvum* infections should help confirm their role in the regulation of plant defense.

F-box-type E3 ligases have been implicated in the regulation of a variety of plant developmental processes [46]. Among the members of the F-box-type E3s implicated in plant defense signaling, the *Coronatine Insensitive1* (*COI1*) gene is the best example from which detailed functional characterization is available. *COI1* encodes a 66-kDa protein with amino-terminal F-box motif and a leucine-rich repeats (LRR) domain close to the carboxy-terminal end [47]. *COI1* is required for plant response to jasmonic acids (JAs), which regulate root growth, pollen fertility, wound healing, and defense against insects and pathogens [48, 49]. The *coi1* mutant fails to express the defense-related gene *plant defensin 1.2* (*PDF1.2*) and is susceptible to insect herbivory and to pathogens [50, 51]. Thus, it is believed that *COI1* plays a positive role in the control of plant defense. Importantly, mutation of the tomato *COI1* homolog *LeCoi1* (also called *jai1*) resulted in deficiency in multiple processes in tomato, including sensitivity to JAs, resistance to herbivores, glandular trichome development, and seed maturation [52], suggesting that *COI1* function is largely conserved among different plant species. The *COI1* protein associated with AtCUL1, AtRbx1, and either of the *Arabidopsis* Skp1-like proteins ASK1 or ASK2 to assemble ubiquitin-ligase complexes SCF<sup>COI1</sup> in controlling JAs responses [53, 54]. It is hypothesized that after activated by the JA signals, the SCF<sup>COI1</sup> E3 ligase targets regulators of JA-signaling pathways for ubiquitination, which leads to either modification of their activity or their degradation. Two putative candidate substrates of the SCF<sup>COI1</sup> E3 ligase have been identified recently and their functional characterization deepened our understanding of *COI1*-dependent plant defense signaling [55, 56]. The histone deacetylase RPD3b (reduced potassium dependency 3b) was found to interact with *COI1* in yeast two-hybrid screens, suggesting that it might be a *COI1* target [55]. Histone deacetylation

is involved in chromatin remodeling and is believed to reduce the transcription of the genes in the region where chromatin is modified [57]. Therefore, it is likely that SCF<sup>COI1</sup>-mediated modification or breakdown of RPD3b will derepress JA-dependent gene expression. In screening for suppressors of the *coi1* mutant phenotype, Xiao *et al.* (2004) identified a mutation called *cos1* (*coi1* suppressor1) that can partly restore JA-related phenotype in the *coi1* mutant [56]. COS1 encodes lumazine synthase, a component of the riboflavin pathway, and could be another putative target of SCF<sup>COI1</sup> E3 ligase. SON1 is another F-box protein that has been implicated in plant defense signaling [58]. The *son1* (*suppressor of nim1-1*) mutant was isolated from genetic screens for suppressors of *non-inducible immunity1* (*nim1*, also referred as *npr1*), of which the corresponding wild-type gene is a key positive regulator of *Arabidopsis* SAR [59, 60]. The *son1* mutant displays SAR-independent, constitutive resistance against both the virulent oomycete *Peronospora parasitica* and the bacterial pathogen *Pseudomonas syringae* pv *tomato* strain DC3000, indicating that SON1-mediated ubiquitination negatively regulate plant defense. The observation that suppression of *SKP1* in tobacco plants by a gene-silencing strategy leads to the loss of *N*-mediated resistance to TMV further supports the notion that F-box proteins and their cognate SCF E3 ligases play a major role in plant defense signaling [61].

### U-box-type E3 ligases are newly identified members of the ubiquitination system involved in plant defense

As discussed earlier, the U-box-containing proteins are newly identified E3 ubiquitin ligases that are present in yeast, plants, animals, and humans [35, 62, 63]. However, the number of U-box proteins varies among different organisms with a larger number of such proteins existing in the plant genomes. Less than 20 U-box proteins were predicted in the human genome [64]. Analyses of the rice fungal pathogen *Magnaporthe grisea* genome identified only two putative U-box proteins (L. Zeng, unpublished). In contrast, 59 and 78 U-box proteins were identified in the *Arabidopsis* (<http://www.arabidopsis.org/info/genefamily/pub.html>) and rice genomes (L. Zeng, unpublished), respectively [65]. The uneven distribution of U-box proteins across different kingdoms prompts the speculation that the major role of plant U-box proteins is to signal responses to biotic and abiotic stresses [64]. However, the diversity of protein domains and motifs presented in rice and *Arabidopsis* U-box proteins argued that these could also play critical roles other than stress control (L. Zeng, unpublished). To date, several U-box proteins have been shown to be involved in plant defense against biotic and abiotic stresses (Table

1). The transcripts of U-box genes *CMPG1* and *CMPG2* were shown to accumulate extremely rapidly in parsley cell cultures after elicitor treatment, and the accumulation was localized around the pathogen infection sites in plants inoculated with *Phytophthora sojae* [66]. *Arabidopsis* homologs of *CMPG1* and *CMPG2* showed a similar expression pattern after challenged by elicitors [67]. In both cases, the authors identified a cis-acting element in the promoters of the genes that showed rapid responsiveness to pathogen but not wounding. The *CMPG* genes encode proteins with an amino-terminal U-box domain and a carboxy-terminal half-rich in leucine residues. Although a rapid activation of these genes in pathogen-treated tissue inferred that they might play an important role in disease resistance, how these genes contribute to plant defenses remains elusive.

In addition to biotic stresses, U-box proteins have also been implicated in plant abiotic stress responses (Table 1). To date, one of the best-studied U-box proteins is CHIP (carboxyl terminus of Hsc70-interacting protein) in mammals. The CHIP protein has an amino-terminal tetratricopeptide repeat (TPR) domain and carboxy-terminal U-box domain. In mammals, the CHIP protein interacts with molecular chaperones such as HSP70 and HSP90 family members and removes substrate proteins of chaperones from the folding pathway to the ubiquitin-proteasome pathway for degradation [68]. CHIP was shown to possess an E3 ubiquitin ligase activity [69, 70] and has been implicated in ubiquitination and proteasomal targeting of a variety of proteins, such as transcription factors SMAD and E2A [71, 72] and nitric oxide synthase [73, 74]. CHIP was also found to be able to promote formation of Lys63-linked polyubiquitin chains in collaboration with Uev1a-Ubc13 *in vitro* [75]. In plants, the *Arabidopsis* CHIP gene *AtCHIP* is upregulated upon temperature stress [76]. However, overexpression of *AtCHIP* in *Arabidopsis* rendered plants more sensitive to both low- and high-temperature treatments, suggesting that CHIP's function in plants is complex and it warrants further detailed characterization.

The rice *spl11* mutant displays a constitutive cell death phenotype in the absence of pathogen challenge and it belongs to a collection of mutants designated as "lesion mimics" based on their spontaneous lesion phenotypes similar to the HR or disease symptoms [77, 78]. The cell death phenotype of *spl11* was shown to be controlled by a single recessive gene [79]. The *spl11* mutant conferred elevated non-race-specific resistance to both *M. grisea* and *Xanthomonas oryzae* pv. *oryzae*, the causal pathogens of rice blast and bacterial blight diseases, respectively [80]. The enhanced resistance of the *spl11* mutant to the two pathogens correlates with the constitutive expression of several defense-related genes and lesion development on

**Table 2** Oxidative stress- or cell death- and defense-related genes with over 4.5-fold upregulation compared with the wild-type plant IR68 at different leaf lesion development stages in *spl11*

Probe sets	Fold upregulation			Protein or homolog
	YL	FL	OL	
<b>Oxidative stress or cell death-related</b>				
os_ORF009142_at	1.0	2.5	4.9	L-ascorbate oxidase
os001035_at	1.3	5.1	6.7	Alcohol dehydrogenase V
os001069_at	0.7	2.8	5.2	Catalase isozyme A
os002264.1_f_at	1.4	5.1	8.8	Alcohol dehydrogenase short chain
os002995_i_at	0.8	2.9	5.1	Alcohol dehydrogenase III
os004902_at	1.2	5.2	4.6	Glutathione-s-transferase
os007966.1_at	1.4	5.1	5.2	Flavanone 3-hydroxylase-like protein
os010415.1_i_at	1.1	27.4	82.2	Putative alcohol dehydrogenase
os013624.1_f_at	1.4	6.0	10.0	Short chain of alcohol dehydrogenase-like
os010304_at	1.0	7.2	5.7	Potassium transporter
os004010.1_at	1.6	5.4	2.9	AAA-type ATPase (yeast BCS1-like)
os000404_at	1.4	7.5	21.1	Bowman-birk-type bran trypsin-like
os006324.1_at	1.1	3.7	4.6	Germin-like protein 1 precursor
<b>Defense-related</b>				
os_ORF008199_at	5.4	8.1	5.5	Extensin precursor
os_ORF011125_at	2.6	5.7	2.5	Endochitinase 1 precursor
os_ORF015958_at	5.2	26.4	7.2	Beta-1, 3-glucanase
os000314_r_at	3.0	28.8	20.5	Rice PR-5
os000349_at	1.8	9.4	7.4	PR-3
os000452_at	1.2	9.3	9.5	Soybean p21 (thamatin-like)
os000536_s_at	1.1	22.9	17.3	PR-1-like
os000590_f_at	1.6	6.2	6.8	Endochitinase precursor
os000608.1_at	1.3	13.9	13.1	Hevein-like protein precursor
os000639_i_at	3.4	37.2	18.1	Beta-1,3-glucanase precursor
os000733_f_at	2.2	7.6	4.1	Phenylalanine ammonia-lyase (PAL)
os000793_f_at	1.6	10.4	7.1	PAL-like
os000816_i_at	1.3	11.0	7.9	PAL-like
os000818_r_at	1.8	10.3	6.5	PR-2
os000915_at	1.2	6.4	6.7	PR-1-like
os000920_s_at	1.2	6.9	4.4	Beta-1, 3-glucanase
os000959.1_at	2.9	10.5	2.0	Beta-1,3-glucanase (isozyme V)
os001265_f_at	1.2	11.5	7.9	PR-1 precursor
os001563.1_at	2.3	21.2	30.1	PBZ1
os002484_f_at	4.5	7.2	5.3	Extensin precursor-like
os002683_at	1.1	35.2	19.6	Subtilisin-chymotrypsin inhibitor-2-likevvv
os002953.1_at	2.0	5.8	3.6	Anthranilate synthase
os003151_f_at	1.0	5.1	6.8	Class IV chitinase
os003773_at	1.7	6.9	4.2	PR-3 (type Q)
os008091_f_at	1.1	1.8	9.1	Basic endochitinase 2 precursor
os011227.1_at	1.3	8.3	2.6	Dehydration-responsive protein RD22-like
os012858_s_at	2.0	5.3	3.6	Anthranilate synthase component II
os015959_at	1.1	4.9	2.8	Beta-glucosidase-like
os016332_i_at	1.0	6.9	11.0	Subtilisin-like protease inhibitor
os_ORF014847_at	1.1	8.5	17.4	Subtilisin-like protease inhibitor
os016724_i_at	1.5	4.1	4.6	Zeamatin
os017465_at	1.1	5.7	8.4	Beta-glucosidase
os024957.1_at	6.2	1.0	0.6	Salt-stress-induced protein-like

the leaves [80]. The constitutive activation of defense responses in the *spl11* mutant was further confirmed when an array-based expression profiling was performed using the Sygenta rice gene chips that harbor nearly half of all rice genes [81] to analyze the impact of the *spl11* mutation on the rice transcriptome. Comparison of the global transcripts between the wild-type plants and the *spl11* mutant plants in three types of leaf tissues with different lesion development phenotypes revealed that the *spl11* mutation causes significant changes in the rice transcriptome (data not shown). Over 300 genes were highly induced in the fully expanded leaves of *spl11*. Nearly half of the highly induced genes are classified as oxidative stress/cell death- or defense-related genes (Table 2 and data not shown). The *Spl11* gene was isolated by a map-based cloning strategy and it encodes a protein with a central U-box domain and carboxy-terminal armadillo (ARM) repeats domain [82, 83]. SPL11 homologs were found in several other plant species but not in human, animals, and yeast, suggesting that SPL11 might be unique to plants. *In vitro* ubiquitination assay indicated that the SPL11 protein possesses E3 ubiquitin ligase activity that is dependent on an intact U-box domain, suggesting a role of the ubiquitination system in the control of plant cell death and defense [83]. We speculate that SPL11 may target one or more regulators that promote PCD and defense signaling pathways. In attempting to identify such substrate proteins and other components potentially involved in *Spl11*-mediated cell death and defense signaling, we have isolated several SPL11-interacting proteins in yeast two-hybrid screens (L. Zeng et al., unpublished). Two of the proteins that showed strong interaction with SPL11 were found to be homologous to pre-mRNA processing proteins, inferring that SPL11 might regulate plant cell death and defense through the control of alternative splicing of the transcripts of cell death and/or defense regulators. The ubiquitination machinery has been demonstrated to be involved in the control of general transcription [84]. But the involvement of ubiquitination in the control of processing specific transcripts has not yet been reported. Functional characterization of the SPL11 interactors should shed light on the molecular basis by which ubiquitination system contributes to plant defense signaling and regulation.

### Other components of the ubiquitination system are also involved in plant defense signaling

Evidence on the involvement of other components in the ubiquitination system in plant defense signaling came from the genetic analysis of the SCF complexes in *Arabidopsis*. Several components that potentially regulate E3 ligase activity were implicated in plant defenses in these studies (Table 1). The RUB (related to ubiquitin, also known as

Nedd8) is a ubiquitin-like protein that covalently attaches to Cullins, the core subunit of the SCF E3 ligase complex. Rub1/Nedd8 and consequently its conjugation pathway are required for the SCF E3 activity [85, 86]. Mutation of *RCE1* (RUB-conjugating enzyme1) resulted in defects in the expression of defense-related genes' basic chitinase and *PDF1.2*, inferring that the SCF E3 complex it modified is required for plant defense [87]. *RCE1* was shown in *Arabidopsis* to be part of a stable SCF<sup>TR</sup> ubiquitin E3 ligase complex [88], which is a critical component in plant auxin signaling [89-91]. It will be intriguing to see if *RCE1* is also associated with SCF<sup>COI1</sup> complex, which has been shown to be involved in plant defense signaling (see above).

The plant *SGT1* (*Suppressor of G2 allele of skp1*) gene was first isolated independently by two laboratories; one in the searching for interacting proteins for the key signaling component of multiple *R*-gene-mediated resistance, *RAR1*[12], and the other in the screening for the genes essential for *R* gene *RPP5*-mediated disease resistance, respectively [92, 93]. Studies from several groups indicated that *SGT1* is required by a diverse range of *R* genes from different plant species in response to a variety of pathogens [61, 92-95]. In yeast, *SGT1* is a key regulatory protein in centromere function and cell cycle transition and is associated with *SKP1* to regulate the activity of the SCF E3 ligase complex [96]. Thus it is speculated that *SGT1* functions in plant defense by regulating the activity of the yet unknown SCF E3 ligase that regulates plant defense signal transduction. This is partly supported by the observation that the *Arabidopsis SGT1* gene could complement yeast *sgt1* mutations and that *SGT1* is associated with SCF subunits *SKP1* and *CUL1* in barley and tobacco cell extracts [61, 93]. The finding that *SGT1* is required for SCF<sup>TR</sup>-mediated plant auxin signaling indicated that it is involved in the regulation of plant physiological processes other than defense responses and also supported the notion that the mechanism by which *SGT1* functions might be conserved across kingdoms [97]. Nevertheless, it is unlikely that SCF<sup>TR</sup> is the E3 ligase related to *SGT1* in the control of *R*-gene-mediated resistance. It is possible that several different F-box proteins and their cognate SCFs associate with *SGT1* and that each controls the signal transduction mediated by different subset of *R* genes. Identification of such F-box protein(s) will be instrumental to an in-depth understanding of the molecular mechanism underlying *SGT1*-regulated, *R*-gene-mediated disease resistance.

At present, it is unclear how *SGT1* regulates the activity of SCF E3 ligase. However, several pieces of evidence might provide some clues to this question. Takahashi *et al.* (2003) demonstrated that barley *SGT1* (*HvSGT1*) and *Arabidopsis SGT1b* (*AtSGT1b*) both interacted with barley cytosolic heat shock protein 90 (*HvHSP90*) in yeast cells



and HvHSP90 co-immuno-precipitated with HvSGT1 in barley plants [98]. The HSP90 proteins are members of the heat shock protein superfamily that are implicated in protein complex maturation, in the activation of innate immune system, and in regulating the activity of many signal transduction proteins [99-101]. In animals, SGT1 was demonstrated to share structural features with HSP90 co-chaperones of the p23 families [102], which prompted the speculation that SGT1 might have a co-chaperone function in facilitating SCF complex assembly. In *Arabidopsis*, mutation of SGT1 did not affect the assembly of the SCF<sup>TIR</sup> complex [97]. This argues that SGT1, rather than facilitating the assembly of the SCF complex, might regulate the SCF activity by other means such as assisting in the ubiquitination process once the substrate is bound. Recent genetic analyses from the Dangl group suggested that *Arabidopsis* SGT1b has two genetically separable functions in some *R*-gene-mediated resistance pathways, being a positive regulator of *R*-gene-induced HR cell death and a negative regulator in pre-activated R protein accumulation, which is antagonized by both RAR1 and HSP90 [103]. They proposed that SGT1b might associate with SCF in targeting HSP90-associated R proteins for ubiquitination-mediated degradation.

The COP9 (constitutively photomorphogenic 9) signalosome (CSN), a conserved multi-protein complex involved in light-dependent plant morphogenesis and other plant developmental pathways, shows remarkable similarity to the lid subcomplex of the 19S RP [104, 105]. It is speculated that CSN might substitute for the lid of the 19S RP in creating new forms of 26S proteasome with distinct specificities [22]. Subunits of the COP9 signalosome are required for N-gene-mediated resistance to TMV [61]. Multiple lines of evidence support the notion that COP9 is a regulator of SCF and other Cullin-based ubiquitin ligases by cleavage of the Nedd8 from the CUL1 of the SCF complex [86, 106, 107], suggesting that COP9 plays its role in plant defense through the ubiquitination system. The interaction of the COP9 signalosome with SGT1 and RAR1 *in vivo* further support this hypothesis [61, 93].

### Pathogen-derived proteins act as E3 ligases in host cells

In the last few years, several studies have demonstrated that some viral proteins act as E3 ligases in host cells. The MIR proteins of Kaposi's sarcoma-associated virus (KSHV) regulate the accumulation and trafficking of target membrane proteins by acting as E3 ubiquitin ligases [108]. The same group recently reported that MIR1, but not MIR2, promoted downregulation of MHC I molecules lacking lysine residues in their intracytoplasmic domain. In

the presence of MIR1, these MHC I molecules were ubiquitinated, and their association with ubiquitin was sensitive to  $\beta$ -mercaptoethanol, unlike lysine-ubiquitin bonds [109]. Similarly, the RING-finger proteins MBP-IAP2, IE2, and PE38 of *Bombyx mori nucleopolyhedrovirus* (BmNPV) are able to reconstitute ubiquitination activity *in vitro* and their activity is dependent on their RING-finger motif [110]. Interestingly, Huang *et al.* [111] demonstrated that the p28 proteins encoded by the ectromelia virus and the variola virus possess E3 ubiquitin ligase activity in biochemical assays as well as in cultured mammalian cells. Point mutations disrupting the RING-finger domain of p28 completely abolish its E3 ligase activity. In addition, p28 functions cooperatively with Ubc4 and UbcH5c, the E2-conjugating enzymes involved in 26S proteasome degradation of protein targets. Whether any plant viral proteins could act as an E3 ligase during infection has yet to be reported.

During the infection process, pathogens deliver various virulence proteins into host cells to modulate diverse biochemical processes in plants [112]. In nature, infection of plants by *Agrobacterium*, a soil-borne plant pathogenic bacterium, causes crown-gall disease [113]. The transfer of *Agrobacterium*-transferred DNA (T-DNA) segment from the bacterial tumor-inducing (Ti) plasmid into the host cell, its integration into the host genome, and the expression of its encoded genes are the major steps of the *Agrobacterium* infection process [113]. The bacterial-transferred (T) DNA is believed to be imported into the host cell nucleus as a DNA-protein complex (T-complex), within which the associated bacterial virulence protein such as VirD2 and VirE2 mediate the nuclear import of the T-DNA [114]. Host proteins, such as VIP1, have also been demonstrated to be involved in the T-DNA-importing process [115]. The associated proteins must be uncoated from T-DNA before it is integrated into plant genome. Recent study showed that VirF, one of the few known exported Vir proteins from *Agrobacterium* is involved in targeted proteolysis of VIP1 and VirE2, probably facilitating the uncoating and integration process [116]. Experiments in yeast and *in planta* revealed that VIP1 and its cognate VirE2 are specifically targeted by the VirF-containing Skp1-Cdc53-cullin-F-box complex for proteolysis. Direct evidence on how the bacterial-secreted proteins act as an E3 ligase in plant cells and affect the host defense response came from a recent study by Janjusevic *et al.* [117]. When the *Pseudomonas syringae*-type III effector AvrPtoB is delivered into tomato plants containing the *Pto* resistance gene, it elicits PCD that inhibits pathogen invasion. In contrast, AvrPtoB suppresses programmed cell death (PCD) in the *Pto*-absent, *Rsb*-containing plants, leading to rapid pathogen spread in the infected tissues. Structural analysis indicated that the C-terminal region of AvrPtoB is highly homologous to the

U-box and RING-finger domain of eukaryotic E3 ligases. *In vitro* E3 ligase activity assay indicated that it has ubiquitin ligase activity. Mutation of the conserved residues in the U-box/RING-finger motif of AvrPtoB significantly compromised AvrPtoB's anti-PCD activity in tomato leaves and dramatically reduced disease symptom on infected plants. These results clearly demonstrated that *P. syringae* use an E3 ligase effector protein to suppress plant PCD and probably other defense-related processes in the infected cells. Identification of the associated E2 enzyme and potential host substrate of AvrPtoB involved in the interaction will be crucial in the future studies.

## Conclusions

In the past few years, we have witnessed the identification of different components of the ubiquitin/26S proteasome system that are involved in plant-pathogen interactions. The emerging picture suggested that ubiquitination modification might occur at different levels of the defense responses in plants, from the R protein itself to downstream signaling components or regulators. In animals, the implication of the ubiquitination system in targeting key regulators in various processes has been well documented. For example, ubiquitination of key pro- and anti-apoptotic regulators was shown to be the principal mechanism underlying the regulation of apoptosis by the ubiquitination/26S proteasome system [118]. In this sense, it will not be surprising that key plant defense signaling components or regulators will be identified as the substrates of the ubiquitination system in the near future. Moreover, the extreme importance of the ubiquitination system in regulating a wide range of cellular processes necessitates fine-tuned spatial and temporal occurrences of such modifications. Thus, understanding of when and where (i.e., at what level) ubiquitination operates in regulating plant defenses could be of equal importance to the determination of which substrates are targeted. It is noteworthy that ubiquitinated substrate proteins not necessarily are targeted for degradation [119]. Mono-ubiquitination or non-canonical ubiquitination might also be employed to fine tune the function of modified substrate proteins in plant-pathogen interactions. The recent exciting finding that plant pathogens deliver E3-like effectors into plant cell raises other intriguing questions such as how these E3 ligase proteins from pathogens interfere with the activation and signaling of host defense responses. Identification and functional characterization of their substrates in the host cells will definitely provide new insights into the molecular mechanisms associated with plant recognition of the pathogen and subsequent activation of defense responses.

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