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Review

Conventional and unconventional ubiquitination in plant immunity

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SUMMARY

Ubiquitination is one of the most abundant types of protein post-translational modification (PTM) in plant cells. The importance of ubiquitination in the regulation of many aspects of plant immunity has been increasingly appreciated in recent years. Most of the studies linking ubiquitination to the plant immune system, however, have been focused on the E3 ubiquitin ligases and the conventional ubiquitination that leads to the degradation of the substrate proteins by the 26S proteasome. By contrast, our knowledge about the role of unconventional ubiquitination that often serves as non-degradative, regulatory signal remains a significant gap. We discuss, in this review, the recent advances in our understanding of ubiquitination in the modulation of plant immunity, with a particular focus on the E3 ubiquitin ligases. We approach the topic from a perspective of two broadly defined types of ubiquitination in an attempt to highlight the importance, yet current scarcity, in our knowledge about the regulation of plant immunity by unconventional ubiquitination.

Keywords: E3 ubiquitin ligase, K63-linked, non-degradative, plant immunity, ubiquitin, unconventional ubiquitination.

INTRODUCTION

Of the many types of protein post-translational modification (PTM), ubiquitination is unique to eukaryotes and is known to be one of the most abundant protein modification processes in the cell (Hershko and Ciechanover, 1998; Khoury *et al.*, 2011; Walsh, 2006). The process of ubiquitination involves covalent attachment of the highly conserved small protein, ubiquitin, to substrate proteins through a stepwise enzymatic cascade that is typically catalysed by three different classes of enzyme: ubiquitin-activating enzyme (E1 or UBA), ubiquitin-conjugating enzyme (E2 or UBC) and ubiquitin ligase (E3) (Callis, 2014). During the ubiquitination process, free ubiquitin is first activated by an E1 enzyme in an ATP-dependent manner, leading to the formation of a thioester bond between the C-terminus of ubiquitin and the catalytic cysteine residue of E1. The activated ubiquitin is then transferred from E1 to the catalytic cysteine residue of an E2 enzyme. In the third step of the cascade, the E2–ubiquitin conjugate cooperates with an E3 to transfer ubiquitin to the substrate (Fig. 1). Activity of the E1–E2–E3 cascade results in the attachment of a single ubiquitin to the substrate protein, typically forming an isopeptide bond between the C-terminus of the ubiquitin and the ϵ-NH_2 group of a substrate lysine residue, which is referred to as monoubiquitination. The enzymatic cascade can occur repetitively on the same substrate, either at multiple additional sites, which is known as multi-monoubiquitination (also as multiubiquitination), or leads to the formation of a polyubiquitin chain (i.e. polyubiquitination) in which, after the first ubiquitin moiety is linked to the substrate protein, the further incoming ubiquitin molecules are linked to the prior ubiquitin moiety (Fig. 2).

The ubiquitin protein contains seven lysine (K) residues that are located at fixed positions of the polypeptide (i.e. K6, K11, K27, K29, K33, K48 and K63). Conventionally, ubiquitination is referred to as the attachment of the lysine-48 (K48)-linked polyubiquitin chain to a substrate protein that serves as the principal signal for protein degradation by the 26S proteasome (Hershko and Ciechanover, 1998). Nonetheless, other types of ubiquitination, including monoubiquitination and polyubiquitination, in which ubiquitin chains are formed through the linkage of lysine residues other than K48 of the ubiquitin molecule, i.e. K6-, K11-, K27-, K29-, K33- and K63-linked ubiquitination, or through the C- or N-terminus of the ubiquitin moieties (i.e. linear ubiquitination), have also been discovered in cells (Komander, 2009). In addition, ubiquitin chains with mixed linkage, such as K63-N-terminus (M) linkage and branched/forked linkage, have also been identified in human and animal cells (Emmerich *et al.*, 2013; Meyer and Rape, 2014). The ubiquitination that occurs in cells is thus highly diverse and complex (Fig. 2). Accordingly, the function, abundance or subcellular distribution of substrate proteins involved in different cellular and physiological processes can be regulated through different types of ubiquitin modification, which leads to distinct fates for these proteins. In addition, ubiquitination can also be reversed by deubiquitination enzymes (DUBs), which add another layer of complexity to the effect of ubiquitination on the fates of

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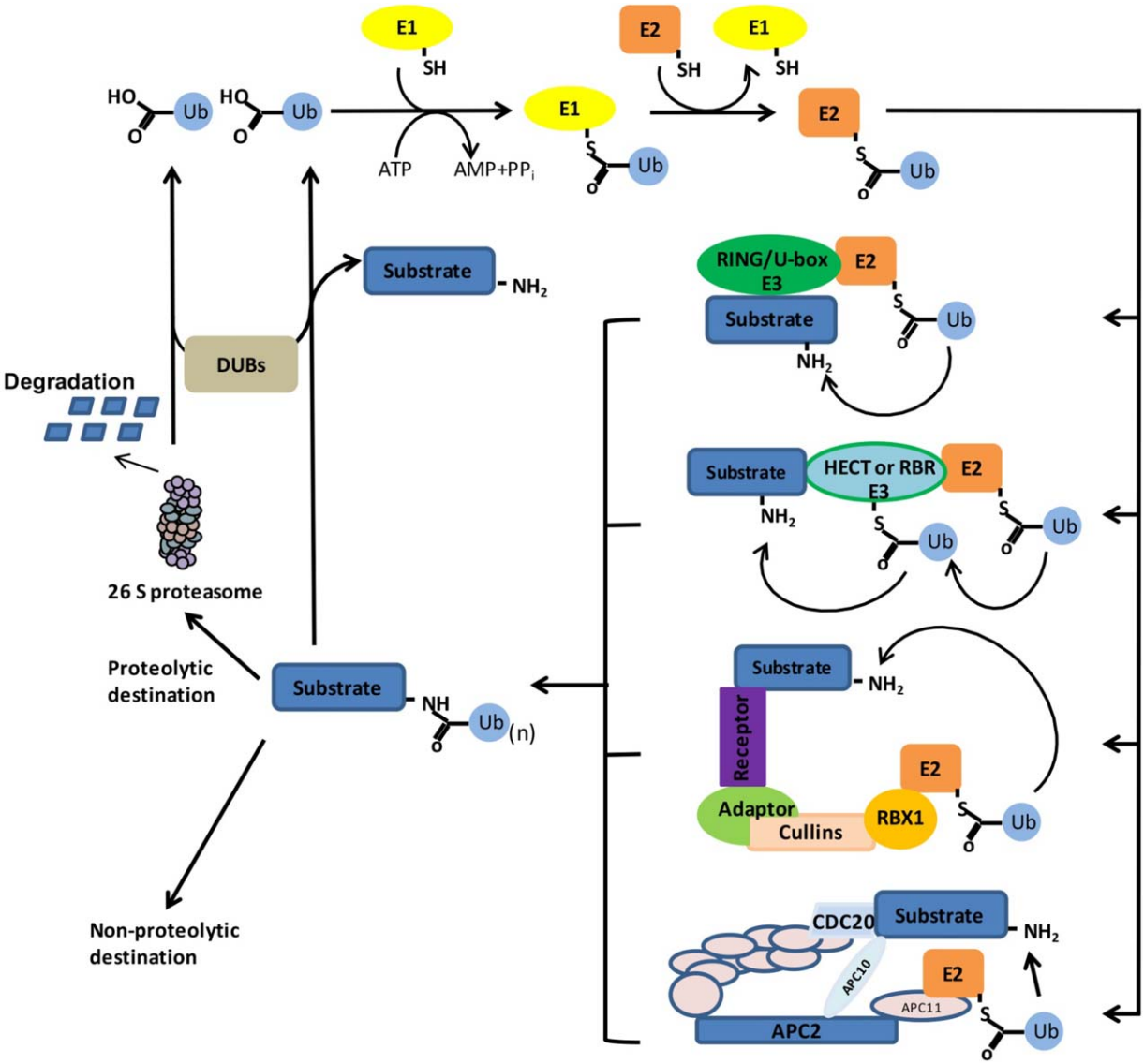

Fig. 1 The ubiquitination cascade and functional consequences of ubiquitination and deubiquitination. Free ubiquitin (Ub) molecules are activated in an ATP-dependent manner by a ubiquitin-activating enzyme (E1). Activated ubiquitin is then transferred to the active site cysteine (Cys) residue of a ubiquitin-conjugating enzyme (E2). The final step of the cascade is mediated by ubiquitin ligase (E3) which recruits the substrate protein in proximity to the ubiquitin–E2 intermediate and facilitates the transfer of the activated ubiquitin typically to the lysine (Lys) residue of the substrate. Depending on the type of E3 involved, the ubiquitin can either be transferred directly from E2 to its substrate (RING, U-box and Cullin-RING E3 ligase) or form a thioester intermediate with E3 before transfer (HECT and RBR E3 ligase). The enzymatic cascade can be repeated after the first ubiquitin is attached to the substrate to form a ubiquitin chain. The type of ubiquitin modification determines the fate of the substrate protein, leading to either degradation by the 26S proteasome or a non-proteolytic process. Deubiquitination enzymes (DUBs) can catalyse the removal of ubiquitin from the conjugated substrates and also generate recycled ubiquitin molecules after the ubiquitinated substrates have been degraded.

substrate proteins (Isono and Nagel, 2014; Katsiarimpa *et al.*, 2013; Komander *et al.*, 2009).

To date, ubiquitination appears to be omnipresent in almost every field of plant research and has been shown to regulate nearly all aspects of plant biology, including plant growth, development and responses to abiotic and biotic stresses (Callis, 2014). Of the various types of ubiquitination, the conventional K48-linked ubiquitination is the best characterized. By contrast, other types of ubiquitination are much less well understood. Nevertheless, available data hitherto suggest that they play key regulatory roles in plants. For example, K63-linked ubiquitination regulates DNA replication and repair, protein synthesis, the iron deficiency response and immune signalling (Hamera *et al.*, 2014; Li and Schmidt, 2010; Mural *et al.*, 2013; Wen *et al.*, 2014; Zang *et al.*,

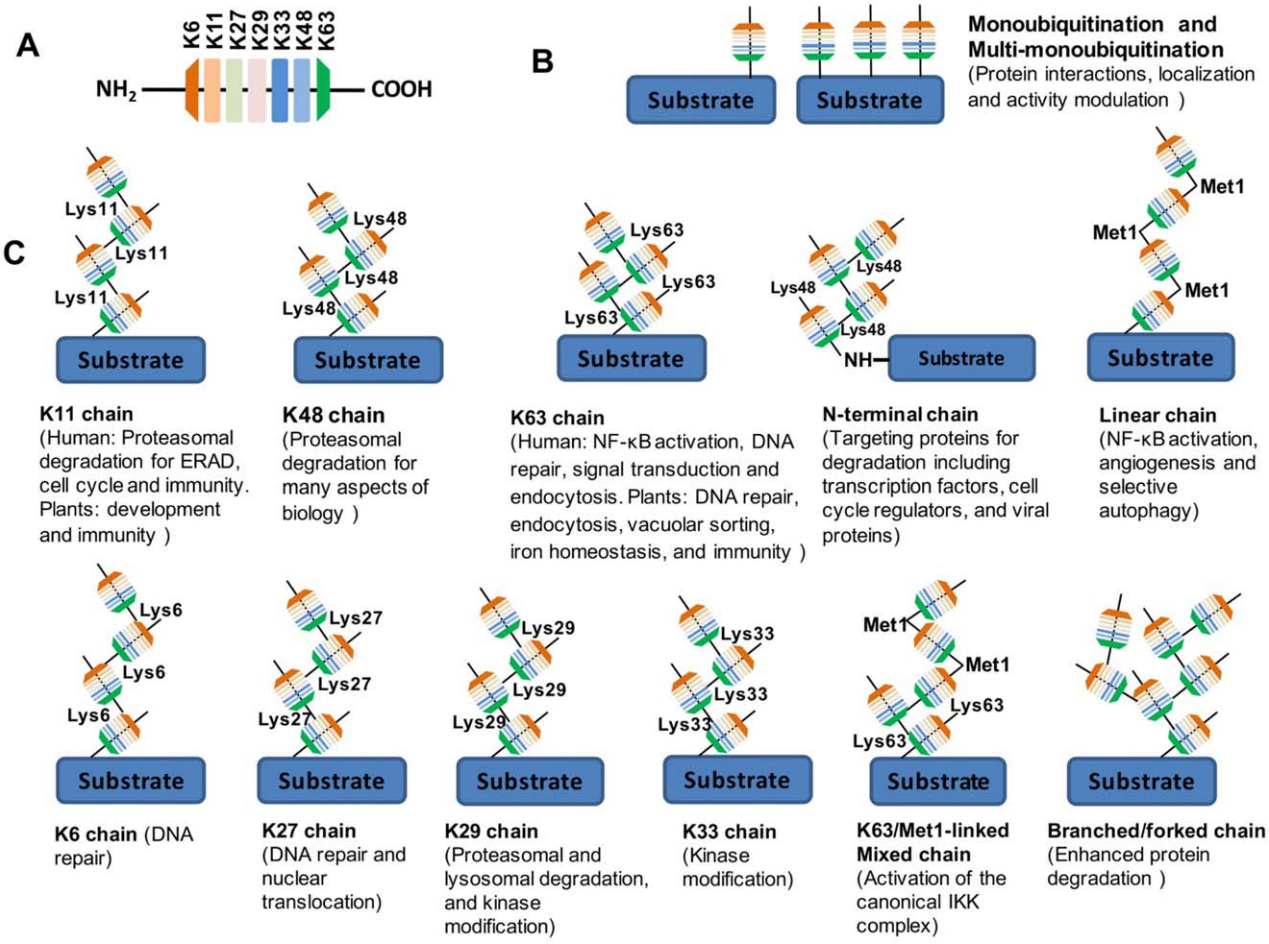

Fig. 2 Diversity of ubiquitination in cells and known roles for different ubiquitin chains. (A) The ubiquitin molecule contains seven lysine (Lys) residues. The carboxyl group of the Gly76 residue of the ubiquitin can form an isopeptide bond linkage to the ϵ-amino group of a Lys residue in the substrate or in another ubiquitin molecule. (B) The substrate can be monoubiquitinated or multi-monoubiquitinated (also known as multiubiquitinated) for the regulation of protein–protein interaction, subcellular localization or activity modulation. (C) Following monoubiquitination, additional ubiquitin moieties can be linked to different Lys residues or to the N-terminal methionine (Met) residue of the prior ubiquitin molecule, resulting in the formation of various types of polyubiquitin chain. Known roles for ubiquitin chains with different linkages are shown. ERAD, endoplasmic reticulum-associated protein degradation. References: monoubiquitination and multi-monoubiquitination (Park and Ryu, 2014; Ye and Rape, 2009); K11 chain (Min *et al.*, 2015; Xu P *et al.*, 2009); K48 chain (Callis, 2014); K63 chain (Mural *et al.*, 2013; Wen *et al.*, 2014; Wu and Karin, 2015); N-terminal chain (Ciechanover and Stanhill, 2014; Sadowski *et al.*, 2012); linear chain (Iwai *et al.*, 2014; Shimizu *et al.*, 2015); K6, K27, K29 and K33 chain (Akutsu *et al.*, 2016; Cunningham *et al.*, 2015; Wong and Cuervo, 2010); K63/Met1-linked mixed chain (Emmerich *et al.*, 2013); branched/forked chain (Ben-Saadon *et al.*, 2006).

2012). Moreover, monoubiquitination of the histone H2B is crucial for the transcriptional regulation of key regulators in plant flowering, seed dormancy and plant immunity (Du *et al.*, 2016; Feng and Shen, 2014; Hu *et al.*, 2014; Zhang Y *et al.*, 2015; Zou *et al.*, 2014). Because many non-K48-linked types of ubiquitination serve as non-proteolytic, regulatory signals in cells, the conventional ubiquitination is thus also often conceived as modification by ubiquitin that leads to the degradation of the substrate proteins by the 26S proteasome. Nevertheless, some unconventional ubiquitination, such as K11-linked ubiquitination, also regulates protein degradation (Xu P *et al.*, 2009).

As sessile organisms that lack a somatically adaptive immune system, plants have developed a sophisticated innate immune system to contend with potential infections by various microorganisms. Conceptually, the plant immune system consists of two layers of defence response, microbe/pathogen-associated molecular pattern (MAMP/PAMP)-triggered immunity (MTI/PTI) and effector-triggered immunity (ETI), depending on the type of pathogen component being recognized (Cui *et al.*, 2015; Jones and Dangl, 2006; Macho and Zipfel, 2014). As in humans and animals, the plant innate immune system is highly regulated and many key mechanisms underlying the regulation have been uncovered (Cui

et al., 2015; Macho and Zipfel, 2014). Notably, the importance of ubiquitination in the regulation of plant immunity has been increasingly appreciated in the past decade. Significant progress regarding the role and underlying molecular basis of ubiquitination in plant immunity has been made. Meanwhile, despite emerging evidence suggesting a critical role for unconventional ubiquitination in the regulation of plant immunity, our knowledge regarding its involvement in plant immunity remains a significant gap. Here, we focus on the recent advances in our understanding of ubiquitination in the modulation of plant immunity, with particular attention given to the E3 ubiquitin ligases. We approach the topic from a perspective of two broadly defined types of ubiquitination and try to highlight the importance, yet current scarcity, in our knowledge of the regulation of plant immunity by unconventional ubiquitination.

THE HIERARCHICAL STRUCTURE OF THE PLANT UBIQUITIN SYSTEM

Like other eukaryotic organisms, a key feature of the plant ubiquitination system (UBS) is its hierarchical structure in terms of the number of ubiquitin E1, E2 and E3 enzymes. A given plant genome usually encodes one or a few E1s, dozens of E2s and over a thousand E3s (Callis, 2014; Du Z *et al.*, 2009; Hatfield and Vierstra, 1992). For example, the Arabidopsis genome has two E1 enzymes, at least 37 ubiquitin E2 enzymes and more than 1500 E3 enzymes (Callis, 2014; Vierstra, 2012). The existence of a large number of E3 ubiquitin ligases reflects well their role as the major governing factor for substrate specificity of the ubiquitination process, i.e. appropriate selection of a myriad of cellular substrate proteins for modification by ubiquitin. As a result of the apparent important function in determining substrate specificity, E3 ubiquitin ligases have been the major interest of research in the characterization of the roles of UBS in the past two decades. By contrast, the roles of E1 and E2 enzymes remain largely unexplored, particularly those that direct unconventional, non-degradative ubiquitination.

The E3 ubiquitin ligases constitute the largest and most diverse group of components of the plant UBS. Members of plant E3 ubiquitin ligases can largely be classified into four subfamilies based on their structural features and mechanisms of action (Fig. 1). The subfamilies HECT (Homologous to E6-associated protein C-Terminus), RING (Really Interesting New Gene) and U-Box, and the recently identified RBR (RING between RING), contain members that are single-subunit proteins. By contrast, members of the subfamily Cullin-RING Ligases are multi-subunit complexes (CRL) (Callis, 2014). The RING finger motif contains Zn^{2+}-chelating amino acid residues typically in the form of C3HC4 (C, cysteine; H, histidine) or C3H2C3, which forms two cross-brace-arranged free loops. This serves as a scaffold that brings the ubiquitin–E2 intermediate and the substrate in proximity to promote the transfer of ubiquitin to the substrate. The U-box contains about 70 amino acids and possesses a tertiary structure resembling that of the RING domain (Ohi *et al.*, 2003). The major difference between the U-box and RING domain is that the U-box lacks the hallmark zinc-chelating cysteine and histidine residues (Ohi *et al.*, 2003). RING and U-box E3s non-covalently interact with the ubiquitin–E2 intermediate to transfer ubiquitin from the intermediate to the substrate protein. By contrast, HECT-type E3s form a covalently linked ubiquitin–E3 intermediate at a highly conserved cysteine residue of the E3 before the ubiquitin is transferred to the substrate. The RBR-type E3s share common features with both the RING and HECT E3 ubiquitin ligase subfamilies. They recruit the ubiquitin–E2 intermediate through its RING domain, but then transfer the ubiquitin to an intrinsic catalytic cysteine residue housed at the C-terminal domain of the RBR E3 ubiquitin ligase prior to its transfer to the substrates, which is similar to the HECT-type mechanism (Spratt *et al.*, 2014). In CRL E3 ubiquitin ligases, the RING domain acts as part of a multi-subunit complex, such as the SCF (Skp1, Cullin1, F-box)-type ubiquitin ligases. The SKP1 protein and F-box-containing protein of the SCF-type E3 ubiquitin ligases work together to bind to the Cullin1 protein, as well as to facilitate the recruitment of specific substrates, whereas the RING protein, e.g. RBX1, binds to the E2 (Choi *et al.*, 2014).

MODULATION OF PLANT IMMUNITY BY E3 UBIQUITIN LIGASES PROBABLY DIRECTING CONVENTIONAL UBIQUITINATION

Findings in the past decade or so have revealed that E3 ubiquitin ligases are involved in various aspects of plant immunity, ranging from the perception of the pathogen to signal transduction and the ensuing immune responses. To our knowledge, all plant ubiquitin E3 enzymes that have thus far been implicated in plant immunity are listed in Table 1. Several reviews summarizing the role of plant ubiquitin ligases in a specific aspect of plant immunity, such as PTI or ETI, have been published (Cheng and Li, 2012; Li *et al.*, 2014; Zeng *et al.*, 2006). Therefore, in this article, we only update the progress that has been made after these reviews became available, and the E3s for which no new findings have been reported in recent years are not discussed. We summarize the new advances based on the subfamilies to which the plant E3s being discussed belong. Some of the plant E3 ubiquitin ligases listed in Table 1 function through the targeting of components of PTI and/or ETI for 26S proteasome-mediated degradation. Ubiquitination catalysed by these E3 ubiquitin ligases is considered as conventional (Fig. 3). However, for many of the E3s shown in Table 1, their substrates related to plant immunity, the feature of ubiquitination they catalyse and the mechanism underlying their involvement in plant immunity remain unknown. It is also noteworthy that a role for the recently identified RBR-type E3s in plant immunity has not yet been uncovered, although this type of E3

Table 1 E3 ubiquitin ligases implicated in plant immunity.

Name	E3 type	Substrate	26S proteasome dependence	Subcellular localization	Role	Target process	References
SPL11/PUB13	U-box	SPIN6, FLS2 and ABI1	Yes	Entire cell	–	PTI, ETI and ABA signalling	Antignani *et al.* (2015), Kong *et al.* (2015), Li *et al.* (2012a), Li *et al.* (2012b), Liu *et al.* (2012b), Liu *et al.* (2015), Lu *et al.* (2011), Monaghan *et al.* (2009), Ning *et al.* (2015), Zeng *et al.* (2004)
PUB22/PUB23/PUB24	U-box	Exo70B2	Yes	Cytosol	–	PTI	Stegmann *et al.* (2012), Trujillo *et al.* (2007)
PUB10	U-box	MYC2	Yes	N	–	JA signalling	Jung *et al.* (2006)
CMPG1/ACRE74	U-box	Unknown	Unknown	N, ER, PM and partially in TGN/EE	+	ETI	Bos *et al.* (2010), Gilroy *et al.* (2011), González-Lamothe *et al.* (2006), Zhu *et al.* (2015)
ACRE276/PUB17/ARC1	U-box	Unknown	Unknown	Nucleus	+	PTI and ETI	He *et al.* (2015), Yang *et al.* (2006)
OsPUB15	U-box	Unknown	Unknown	Cytosol	+	PTI and ETI	Park *et al.* (2011), Wang *et al.* (2015)
OsPUB44	U-box	Unknown	Unknown	Entire cell	+	PTI	Ishikawa *et al.* (2013)
PRP19/MAC3	U-box	Unknown	Unknown	N	+	ETI	Monaghan *et al.* (2009), Xu *et al.* (2012)
XB3	RING	Unknown	Unknown		+	ETI/PTI	Huang *et al.* (2013), Wang *et al.* (2006)
BBI1	RING	Unknown	Unknown		+	PTI and cell wall defence	Li *et al.* (2012a)
APIP6	RING	AvrPiz-t	Unknown		+	PTI	Park *et al.* (2012)
APIP6	RING	OsELF3-2	Yes		+	PTI	Ning *et al.* (2015)
APIP10	RING	AvrPiz-t and Piz-t	Yes		+	PTI	Park *et al.* (2016)
EIRP1	RING	VpWRKY11	Yes	N	+	PTI	Yu *et al.* (2013)
BOI1	RING	BOS1	Yes	N	+	Pathogen and abiotic stress-induced necrosis	Luo *et al.* (2011)
BRGs	RING	Unknown	Unknown		+	Pathogen and abiotic stress-induced necrosis	Luo *et al.* (2011)
RING1	RING	Unknown	Unknown	PM	+	ETI, SA and ABA signalling	Ghannam *et al.* (2016), Lee *et al.* (2011), Lim *et al.* (2015), Lin *et al.* (2015)
RIN2/RIN3	RING	Unknown	Unknown	PM	+	ETI	Kawasaki *et al.* (2013)
KEG	RING	Unknown	Unknown	TGN/EE	+	Intracellular trafficking processes	Gu and Innes (2012), Wawrzynska *et al.* (2008)
ATL1	RING	Unknown	Unknown	TGN/EE	+	ETI	Serrano *et al.* (2014)
ATL31	RING	14-3-3 proteins	Yes	PM	+	C/N stress response and PTI	Maekawa *et al.* (2012), Sato *et al.* (2011), Yasuda *et al.* (2014)
ATL6	RING	Unknown	Unknown	PM	+	C/N regulation, PTI and JA signalling	Hondo *et al.* (2007), Maekawa *et al.* (2012)
ATL9	RING	Unknown	Unknown	ER	+	PTI	Berrocal-Lobo *et al.* (2010)
RFP1	RING	βC1	Yes	N and cytoplasm	+	Anti-*virus*	Shen *et al.* (2016)
MIEL1	RING	MYB30	Yes	N	–	ETI	Marino *et al.* (2007)
BAH1/NLA	RING	Unknown	Unknown		–	ETI and SA signalling	Yaeno and Iba (2008)
RGLG3 and RGLG4	RING	Unknown	Unknown		–	JA signalling	Zhang *et al.* (2012)
HUB1/HUB2	RING	H2B	Monoubiquitination		+/–	Immunity-associated gene expression	Zhang Y *et al.* (2015), Zou *et al.* (2014)
UPL5	HECT	WRKY53	Unknown	Cytoplasm	–	PTI and SA signalling	Chujo *et al.* (2007), Miao and Zentgraf (2010),

Table 1 *Continued*

Name	E3 type	Substrate	26S proteasome dependence	Subcellular localization	Role	Target process	References
							Van Eck *et al.* (2014), Zhang *et al.* (2012)
SCF^{SON1}	CRL	Unknown	Unknown		–	SAR-independent defence response	Kim and Delaney (2013)
$CRL3^{NPR3/NPR4}$	CRL	NPR1	Yes		+	ETI and SA signalling	Matsushita *et al.* (2013), Spoel *et al.* (2009), Wu *et al.* (2012)
$CRL3^{SR1IP1}$	CRL	AtSR1	Yes		+	Calcium-mediated signalling and SA signalling	Zhang *et al.* (2014)
$SCF^{CRP1\text{-}MUSE13/14}$	CRL	NLRs	Yes	Cytosol and PM	–	R-gene-mediated resistance	Huang *et al.* (2012)
SCF^{CRP1}	CRL	SNC1 and RPS2	Yes	Cytoplasm and N	–	R-gene-mediated resistance	Gou *et al.* (2006)
$SCF^{SGT1\text{-}HSP90}$	CRL	SNC1	Yes	Cytosol	–	R-gene-mediated resistance	Huang *et al.* (2014)
$CRL3^{MATH\text{-}BTB}$	CRL	ATHB6	Yes	N	+	ABA signalling	Lechner *et al.* (2015)
SCF^{COI1}	CRL	JAZ proteins	Yes		+	JA signalling	Kazan and Manners (2013), Thines *et al.* (2003)
$CUL4^{COP1}$	CRL	HRT	Yes	N and PM	–	Anti-*virus*	Jeong *et al.* (2007)
$CUL4^{HP1/DDB1}$	CRL	Unknown	Unknown		+	PTI and SA signalling	Liu *et al.* (2008)
APC/C	CRL	CYCB1;1	Unknown		–	R-gene-mediated resistance	Bao *et al.* (2013)
SCF^{ACIF1}	CRL	Unknown	Unknown		+	ETI and N-gene-mediated resistance	van den Burg *et al.* (2007)
SCF^{OsDRF1}	CRL	Unknown	Unknown		+	SA signalling	Cao *et al.* (2008a)

ABA, abscisic acid; C/N, carbon/nitrogen; ER, endoplasmic reticulum; ETI, effector-triggered immunity; JA, jasmonic acid; N, nucleus; PM, plasma membrane; PTI, pathogen-associated molecular pattern (PAMP)-triggered immunity; SA, salicylic acid; SAR, systemic acquired resistance; TGN/EE, trans-Golgi network/early endosome; +, positive; –, negative.

ubiquitin ligase is vital to the regulation of human and animal immune signalling (Elton *et al.*, 2015; Smit and Sixma, 2014).

RING TYPE

The avirulence effector protein AvrPiz-t of the rice fungal pathogen *Magnaporthe oryzae* is recognized by the nucleotide-binding domain and leucine-rich repeat-containing (NLR) immune receptor/resistance (R) protein Piz-t, which leads to the resistance of rice plants to the pathogen (Li *et al.*, 2009). AvrPiz-t has been found to translocate into rice cells to suppress host PTI through a decrease in the activity of a rice RING-type E3 ubiquitin ligase AVRPIZ-T INTERACTING PROTEIN 6 (APIP6) (Park *et al.*, 2012). As a mechanism of counterattack by the host, rice APIP6 is able to target AvrPiz-t for ubiquitination and subsequent degradation, which reduces the suppression of rice PTI by AvrPiz-t (Ning *et al.*, 2015; Park *et al.*, 2012). OsELF3-2, one of the two homologues of Arabidopsis EARLY FLOWERING3 (ELF3) (Yu *et al.*, 2008), negatively regulates rice immunity against *M. oryzae* (Ning *et al.*, 2015). APIP6 also interacts with and targets OsELF3-2 for 26S proteasome-mediated degradation, suggesting that APIP6 plays a positive role in rice immunity by negatively modulating OsELF3-2. Similar to APIP6, another rice RING-type E3 ubiquitin ligase, APIP10, also targets AvrPiz-t for degradation (Park *et al.*, 2016). The study by Park *et al.* (2016) also found that knocking down of APIP10 in the non-Piz-t background compromises the basal defence against *M. oryzae* but, in rice plants that express *Piz-t*, APIP10 attenuates cell death triggered by Piz-t–AvrPiz-t recognition through the promotion of degradation of Piz-t, which antagonizes the effect of AvrPiz-t that stabilizes Piz-t. APIP10 thus apparently plays a positive role in PTI, but functions as a negative regulator of Piz-t-mediated ETI in rice.

The pepper (*Capsicum annuum*) RING E3 ubiquitin ligase CaRING1 is localized in the plasma membrane and induced by *Xanthomonas* infection (Lim *et al.*, 2015). The characterization of plants in which *CaRING1* is silenced or overexpressed suggests that CaRING1 acts as a positive regulator of defence responses (Lee *et al.*, 2011; Lin *et al.*, 2008). Recently, CaRING1 has also been found to be involved in abscisic acid (ABA)-mediated plant responses to dehydration (Lim *et al.*, 2015). The expression of a

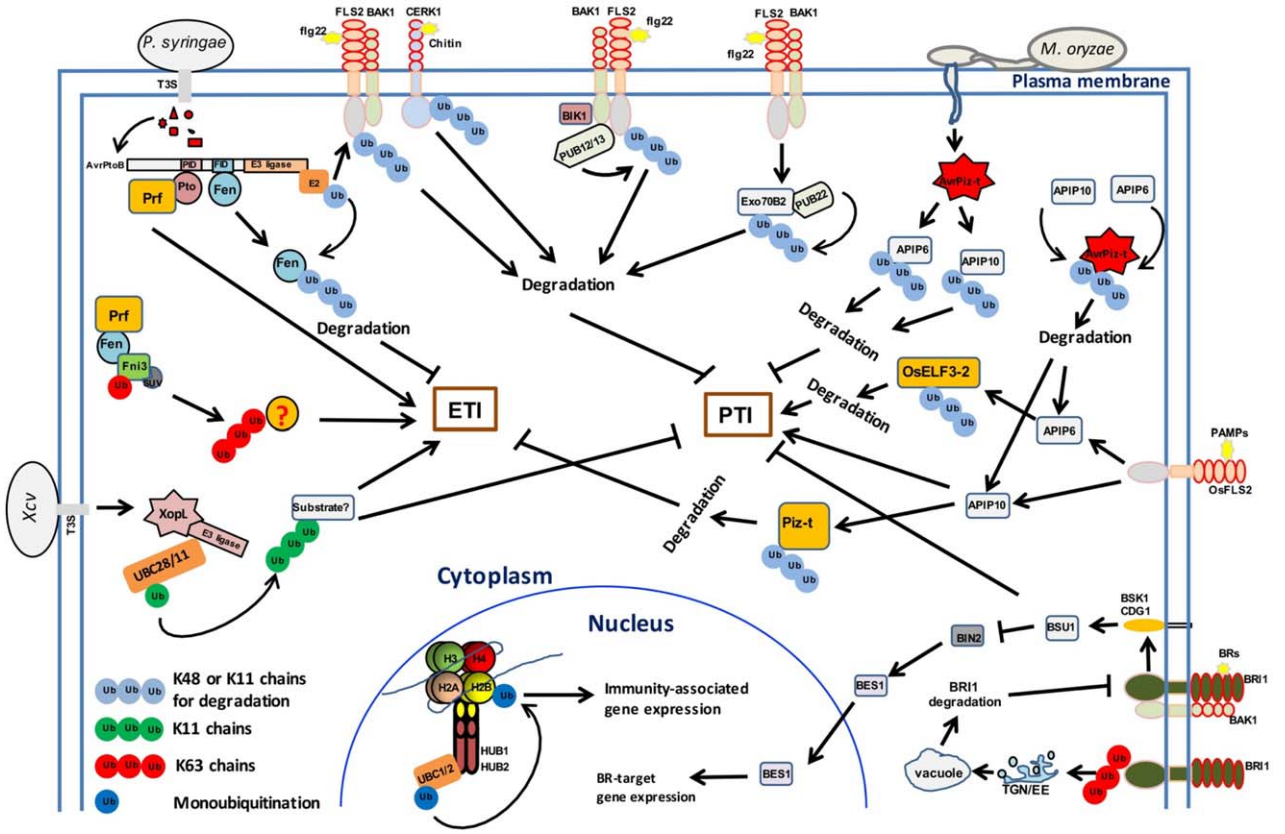

Fig. 3 Examples of the regulation of plant immunity by conventional and unconventional ubiquitination. The conventional K48-linked ubiquitination usually targets substrate proteins for 26S proteasome-dependent degradation, as in several examples shown in this figure. The U-box proteins PUB12/PUB13 and PUB22 attenuate pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) signalling by targeting the pattern recognition receptor FLS2 and the exocyst complex subunit Exo70B2, respectively, for 26S proteasome-dependent degradation. During the co-evolution between pathogens and plants, pathogens have evolved effectors to modulate the host ubiquitin system to subvert plant immunity. The avirulence effector AvrPiz-t from *Magnaporthe oryzae* targets the positive PTI regulators, APIP6 and APIP10, for degradation to suppress PTI in rice. As a mechanism of counterattack by the plant, APIP6 and APIP10 ubiquitinate AvrPiz-t, leading to the degradation of the effector to promote host immunity. In addition, APIP6 ubiquitinates the negative immunity regulator OsELF3-2 to promote PTI, and APIP10 functions as a negative regulator of the R protein Piz-t-mediated effector-triggered immunity (ETI). Two other bacterial effectors, AvrptoB and XopL, exhibit C-terminal ubiquitin ligase activity. AvrPtoB ubiquitinates the receptor proteins FLS2 and CERK1, as well as the protein kinase Fen, for degradation to suppress PTI and ETI, respectively. XopL specifically interacts with plant ubiquitin E2 enzymes, AtUBC28 and AtUBC11, to mediate the formation of predominantly K11-linked polyubiquitin chains to target as yet unknown substrate(s). In the regulation of plant immunity by unconventional ubiquitination, K63-linked ubiquitination has been shown to be involved in Fen-mediated immunity. However, it is unknown at present how FNI3/SUV-directed K63-linked ubiquitination regulates Fen-mediated immunity. K63-linked polyubiquitination also functions as a dual targeting signal for BRI1 internalization and sorting along the endocytic pathway to remove the suppression effect of PTI by brassinosteroid (BR) signalling. The Arabidopsis ubiquitin E2 AtUBC1/AtUBC2 and RING-type E3 ligases HUB1/HUB2 together mediate the monoubiquitination of histone H2B to tune plant immunity through an effect on the expression of plant immunity-associated genes. BIK1, botrytis-induced kinase 1; BSK1, BR-signalling kinase 1; BSU1, BRI1 suppressor protein 1; CDG1, constitutive differential growth 1; CERK1, chitin elicitor receptor kinase 1; TGN/EE, trans-Golgi network and early endosome; Ub, ubiquitin.

tobacco gene encoding the E3 ubiquitin ligase *Nicotiana tabacum* RING PROTEIN 1 (NtRING1) (not homologous to CaRING1) was up-regulated on induction of the hypersensitive response (HR) by elicitin, *Ralstonia solanacearum* or *Tobacco mosaic virus* (TMV). By contrast, silencing of *NtRING1* remarkably delayed the establishment of the elicitin-induced HR and the expression of early HR-induced genes in tobacco, suggesting a role of NtRING1 in the early establishment of HR (Ghannam *et al.*, 2016).

The Arabidopsis RING E3 ubiquitin ligase KEEP ON GOING (KEG) is localized to the trans-Golgi network and early endosomes (TGN/EE) and has been shown to be a key component of TGN/EE that regulates multiple post-Golgi trafficking events in plants, including the targeting of membrane-associated proteins to the vacuole (Gu and Innes, 2012). Loss of function in KEG blocks the secretion of the apoplastic defence proteins PR1 and C14, which implicates KEG in plant immunity. Moreover, KEG is degraded

specifically in cells infected by the fungus *Golovinomyces cichoracearum*, suggesting that the fungal pathogen may promote its virulence by the manipulation of the host secretory system and further implying the involvement of KEG in host immunity (Gu and Innes, 2012). KEG has also been found to interact with ABA BINDING FACTOR 1, 3 and 5 (ABF1, ABF3, ABF5) to negatively regulate ABA signalling (Chen *et al.*, 2013; Stone *et al.*, 2006). More recently, KEG has been shown to act as a positive regulator of stability for JASMONATE ZIM-DOMAIN12 (JAZ12), a representative JAZ protein that interacts with the SCF^{COI1} E3 ubiquitin ligase in a jasmonic acid–isoleucine (JA–Ile)-dependent manner (Pauwels *et al.*, 2015). These findings highlight KEG as an important positive regulator of plant immunity and a novel point of cross-talk between phytohormones ABA and JA. KEG is physically associated with the kinase domain of ENHANCED DISEASE RESISTANCE1 (EDR1) and mediates the TGN/EE localization of EDR1 (Gu and Innes, 2011). Loss-of-function mutations in EDR1 confer enhanced programmed cell death (PCD) under a variety of abiotic and biotic stress conditions, but missense mutations in KEG fully suppress known *edr1* mutant phenotypes (Frye and Innes, 1998; Frye *et al.*, 2001; Wawrzynska *et al.*, 2008). KEG is thus required for *edr1* mutant phenotypes and intimately connected to EDR1 in modulating host immunity, although the molecular characteristic of this connection is currently unknown. In this regard, it would be intriguing to determine whether KEG modifies EDR1 by ubiquitination.

EDR1 also interacts with the ubiquitin ligase ARABIDOPSIS TOXICOS EN LEVADURA1 (ATL1) and negatively regulates its E3 activity (Serrano *et al.*, 2014). In Arabidopsis, the *ATL* gene family encodes 91 plant-specific putative RING-type ubiquitin ligases that contain a transmembrane domain (Aguilar-Hernandez *et al.*, 2011). Overexpression of *ATL1* in tobacco and Arabidopsis induces cell death which is suppressed by EDR1. In addition, knockdown of *ATL1* expression suppresses cell death phenotypes associated with the *edr1* mutant and makes Arabidopsis plants hypersusceptible to powdery mildew infection. ATL1 thus has a positive role in immunity-associated PCD (Serrano *et al.*, 2014). Another two ATL proteins, ATL31 and its closest homologue ATL6, are membrane-associated ubiquitin ligases that are involved in the carbon/nitrogen (C/N) response, and ATL31 regulates the stability of 14-3-3 proteins through ubiquitination (Sato *et al.*, 2009, 2011). Both of these C/N response regulators are involved in the plant immune response (Maekawa *et al.*, 2012). Overexpression of *ATL31* and *ATL6* enhances flg22-induced PTI and defences against *Pseudomonas syringae* pv *tomato* (*Pst*) strain DC3000 (Maekawa *et al.*, 2012), suggesting a positive role of ATL31 and ATL6 in plant immunity. A recent report has shown that ATL31 also controls papilla formation in response to powdery mildew fungus penetration by interacting with SYNTAXIN OF PLANTS121 (SYP121) in Arabidopsis (Maekawa *et al.*, 2014). Another Arabidopsis ATL protein, ATL9, is localized to the endoplasmic reticulum and is implicated in chitin- and NADPH oxidase-mediated defence responses (Berrocal-Lobo *et al.*, 2010). The expression pattern of *ATL9* correlates with basal defence responses against *G. cichoracearum*, a biotrophic fungal pathogen.

The βC1 protein encoded by the *Tomato yellow leaf curl China virus*-associated β-satellite functions as a pathogenicity determinant (Shen *et al.*, 2016). βC1 forms a complex with ASYMMETRIC LEAVES 1 (AS1) to execute its pathogenic functions and has been found previously to suppress a subset of JA responses (Yang *et al.*, 2008). A tobacco (*Nicotiana benthamiana*) RING-type E3 ubiquitin ligase, NtRFP1, has been shown recently to attenuate disease symptoms caused by βC1 protein through the ubiquitination of βC1, which was subsequently degraded via the 26S proteasome (Shen *et al.*, 2016). Plants overexpressing NtRFP1 developed attenuated symptoms against viral infection, whereas plants with silenced *NtRFP1* showed severe symptoms (Shen *et al.*, 2016). These findings together suggest that NtRFP1 probably targets βC1 for degradation and thus diminishes the βC1-mediated suppression of JA, which attenuates disease symptoms. However, this remains to be confirmed.

Unlike the plant RING-type E3 ubiquitin ligases mentioned above which play a positive role in plant immunity, the Arabidopsis RING-type E3 ubiquitin ligases, RING DOMAIN LIGASE3 (RGLG3) and RGLG4, negatively regulate plant immunity. RGLG3 and RGLG4 have been found to be essential upstream modulators of JA signalling in response to methyl jasmonate, *Pst* strain DC3000 and wounding in a coronatine-insensitive protein 1 (COI1)-dependent manner (Zhang *et al.*, 2012). The *rglg3rglg4* mutations attenuate the inhibitory effect of JA–Ile-mimicking coronatine on root elongation and the *rglg3rglg4* mutant is resistant to the coronatine-secreting pathogen *Pst* DC3000 (Zhang *et al.*, 2012). These findings suggest that RGLG3 and RGLG4 act in response to coronatine and promote JA-mediated pathogen susceptibility. The Arabidopsis genome encodes a small family containing five RGLG homologues. RGLG1 and RGLG2 have been shown previously to be involved in the auxin response (Yin *et al.*, 2007). The members of the RGLG family thus seem to be closely associated with plant hormone signalling.

U-BOX TYPE

The U-box-type E3 ubiquitin ligases SPL11/PUB13 have been well characterized in plant immunity. The loss-of-function mutant of the rice SPL11 E3 ubiquitin ligase, *spl11*, displays broad-spectrum resistance to rice bacterial and fungal pathogens (Yin *et al.*, 2000; Zeng *et al.*, 2004). PUB13, the Arabidopsis orthologue of rice SPL11, also negatively regulates PCD and resistance to biotrophic pathogens (Li *et al.*, 2012a,b), indicating that SPL11-like proteins are functionally conserved in monocot and dicot plants. The E3

ubiquitin ligases PUB12 and PUB13 have been further shown to attenuate PTI triggered by host perception of the immunogenic fragment of the bacterial flagellin, flg22 (Lu *et al.*, 2011). PUB12/13 ubiquitinates FLAGELLIN SENSING 2 (FLS2, the immune receptor of flg22/flagellin), leading to the degradation of FLS2 by the 26S proteasome. A recent report has found that a Rho GTPase-activating protein SPL11-INTERACTING PROTEIN 6 (SPIN6) interacts with SPL11 and OsRac1, and negatively regulates PCD and innate immunity in rice (Liu *et al.*, 2015). The rice RING-type E3 ubiquitin ligases HISTONE MONOUBIQUITINATION1 (OsHUB1) and OsHUB2 interact with SPIN6 and form homo- and heterodimers in rice, suggesting that OsHUB1 and OsHUB2 may be associated with the SPIN6/OsRac1 pathway in rice immunity (Ning *et al.*, 2015). In Arabidopsis, RabA4B specifically interacts with the closely related lipid kinases PHOSPHATIDYLINOSITOL 4-KINASE β1 (PI4Kβ1) and PI4Kβ2, and is recruited to vesicles that emerge from the TGN compartments (Kang *et al.*, 2011; Preuss *et al.*, 2004, 2006). RabA4B regulates polarized membrane trafficking in plant cells. PUB13 has been found recently to co-localize with RabA4B to TGN and Golgi compartments. PUB13 has also been found to interact with RabA4B through N-terminal domains and with phosphatidylinositol 4-phosphate (PI-4P) through a C-terminal armadillo domain. PUB13, PI4Kβ1 and PI4Kβ2 negatively regulate salicylic acid (SA)-mediated induction of pathogenesis-related gene expression, which highlights their role in SA-dependent defence signalling (Antignani *et al.*, 2015). In addition, the Arabidopsis phosphatase 2Cs (PP2Cs) are ABA co-receptors that block ABA signalling by inhibition of the downstream protein kinases. On perception of the ABA signal, PP2Cs are inhibited by ABA-bound PYR/PYL/RCAR ABA receptors (PYLs), thus activating ABA signalling. A recent report has indicated that PUB12 and PUB13 modulate a key negative regulator of the ABA signalling pathway, ABA-INSENSITIVE 1 (ABI1, a PP2C protein). PUB12 and PUB13 interact with ABI1, but ubiquitinate ABI1 only in the presence of PYLs in an *in vitro* assay. Both degradation of ABI1 and the ABA-triggered plant responses are reduced in *pub12 pub13* mutants compared with the wild-type. Consistently, introduction of the loss-of-function mutation *abi1-3* into the *pub12 pub13* mutant recovers the ABA-insensitive phenotypes of the *pub12 pub13* mutant (Kong *et al.*, 2015).

Similar to PUB12/13, PUB22, PUB23 and PUB24 also act as negative regulators of PTI triggered by several different PAMPs in Arabidopsis (Trujillo *et al.*, 2008). A follow-up study indicated that PUB22 targets the exocyst complex subunit Exo70B2 for its turnover to regulate Arabidopsis PTI (Stegmann *et al.*, 2012). The *pub22/23/24* triple mutant has also been shown to display increased resistance against the root-infecting fungal pathogen *Fusarium oxysporum*, also suggesting a role of PUB22/23/24 in root-mediated defences against soil-borne pathogens (Chen *et al.*, 2014).

In addition to PTI responses, U-box E3 is also involved in plant immunity-associated JA signalling. The basic helix–loop–helix (bHLH) transcription factor MYC2 has emerged in recent years as a master regulator of many aspects of the JA signalling pathway and as a key point of cross-talk between the signalling pathways of JA and other phytohormones, such as ABA, SA, gibberellins (Gas) and auxin (IAA) (Kazan and Manners, 2013). A recent report has shown that MYC2 is targeted by the U-box-type E3 ubiquitin ligase PUB10 for ubiquitination and subsequent degradation during the JA response (Jung *et al.*, 2015), suggesting that PUB10 negatively regulates the JA signalling pathway. However, the plant immunity-related phenotype on the PUB10 loss-of-function and PUB10-overexpressing plants was not characterized in the report.

In contrast with a negative role for the U-box-type E3 ubiquitin ligases discussed above, the U-box E3 ubiquitin ligase CMPG1 plays a positive role in host immunity-associated PCD. The parsley (*Petroselinum crispum*) and Arabidopsis *CMPG1* genes, *PcCMPG1* and *AtCMPG1*, show immediate to early transcriptional induction on infection or treatment with a pathogen-derived elicitor (Heise *et al.*, 2002; Kirsch *et al.*, 2001). The tobacco homologue of CMPG1, NtCMPG1, has been found to be essential for AvrPto/Pto-, Inf1- and Avr9/Cf-9-mediated HR and Cf9-mediated resistance to *Cladosporium fulvum* (González-Lamothe *et al.*, 2006). CMPG1-V, a homologue of CMPG1 in the diploid wheat relative, *Haynaldia villosa* L., confers a broad-spectrum resistance to powdery mildew (Zhu *et al.*, 2015), which suggests that CMPG1 also participates in the regulation of plant innate immunity in monocots. A separate study, however, has indicated that the effector protein AVR3a of the oomycete pathogen *Phytophthora infestans* interacts with and stabilizes the potato U-box E3 ubiquitin ligase CMPG1 to suppress Inf1-, Avr9/Cf-9-, Avr4/Cf-4-, AvrPto/Pto- and the oomycete pathogen-associated PAMP, CELLULOSE-BINDING ELICITOR LECTIN (CBEL)-triggered, CMPG1-dependent PCD (Bos *et al.*, 2010; Gilroy *et al.*, 2011). Silencing of *CMPG1* reduced *P. infestans* sporulation and lesion development on *N. benthamiana* leaves, supporting the notion that CMPG1 is a virulence target of Avr3a and that E3 activity of CMPG1 might be critical for the necrotrophic stage of pathogen infection.

Similar to CMPG1, tomato and tobacco AVR9/CF-9 RAPIDLY ELICITED 276 (ACRE276, also called PUB17) and its Arabidopsis functional orthologue, the U-box E3 ubiquitin ligase AtPUB17, have been shown to be required for the activation of immunity-associated cell death and act as positive regulators of plant disease resistance (Yang *et al.*, 2006). The potato PUB17 is required for resistance to *P. infestans* and acts in the nucleus to promote specific immune pathways triggered by *P. infestans* (He *et al.*, 2015). Silencing of *PUB17* in tobacco attenuated PTI triggered by flg22 and Avr4/CF4-mediated PCD, but did not compromise cell death triggered by the *P. infestans* PAMP INF1 or co-expression of

AVR3a/R3a, indicating that PUB17 is required for some of the plant immune responses only (He *et al.*, 2015).

Two rice U-box-type E3s, OsPUB15 and OsPUB44, have also been identified to positively regulate plant immunity. OsPUB15 has been shown previously to reduce cellular oxidative stress during seedling establishment (Park *et al.*, 2011). It has been found recently to be a binding partner of the rice blast resistance protein PID2 (Wang *et al.*, 2015). The kinase domain of PID2 is capable of phosphorylating OsPUB15. Rice plants overexpressing *OsPUB15* display spontaneous cell death, excessive accumulation of reactive oxygen species (ROS), increased expression of pathogenesis-related genes and enhanced resistance to different strains of the rice blast pathogen (Wang *et al.*, 2015). OsPUB44 positively regulates peptidoglycan (PGN)- and chitin-induced immunity and resistance of rice to the bacterial pathogen *Xanthomonas oryzae* pv *oryzae* (*Xoo*) (Ishikawa *et al.*, 2014). To promote its virulence, the *Xoo* effector $XopP_{Xoo}$ interacts with the U-box domain of OsPUB44 and inhibits its E3 ubiquitin ligase activity. The interaction of OPUB44 with $XopP_{Xoo}$ is specific, as $XopP_{Xoo}$ shows no interaction with OsPUB45 and OsPUB46, two close homologues of OsPUB44 (Ishikawa *et al.*, 2014).

HECT TYPE

The Arabidopsis genome encodes seven HECT-type E3 ubiquitin ligases which are termed UBIQUITIN PROTEIN LIGASES (UPLs) (Callis, 2014; Downes *et al.*, 2003). The only known HECT E3 ubiquitin ligase likely to be involved in plant immunity is UPL5, which was first identified by a yeast two-hybrid screen to interact with WRKY53, a transcription factor acting positively in leaf senescence (Miao and Zentgraf, 2010). UPL5 is able to ubiquitinate WRKY53 *in vitro*, and overexpression of UPL5 in *upl5* plants leads to degradation of the WRKY53 protein, suggesting that UPL5 may regulate leaf senescence in Arabidopsis through the degradation of WRKY53 (Miao and Zentgraf, 2010). WRKY53 has been implicated in multiple plant physiological processes, including plant responses to biotic and abiotic stress (Murray *et al.*, 2007; Sun and Yu, 2015; Van Eck *et al.*, 2010, 2014). In Arabidopsis, WRKY53 acts as a positive regulator of basal resistance against *P. syringae*, and WRKY46 coordinates with WRKY70 and WRKY53 to confer such basal resistance (Hu *et al.*, 2012; Murray *et al.*, 2007). Rice WRKY53 (OsWRKY53) has been found to positively modulate resistance to various pathogens, such as the rice blast pathogen *M. oryzae* (Chujo *et al.*, 2007). These data suggest a link between UPL5-mediated degradation of WRKY53 and plant immunity. However, direct evidence showing the involvement of UPL5 in plant immunity remains to be discovered.

CRL TYPE

The CRL-type E3 ubiquitin ligases probably constitute the largest group of E3 ubiquitin ligases in plants. For example, the Arabidopsis and rice genomes each encode nearly 700 F-box proteins (Gagne *et al.*, 2002; Jain *et al.*, 2007), not to mention other CRL E3s. The Arabidopsis genome encodes six cullin-like proteins, CULLIN1 (CUL1), CULLIN2, CULLIN3a (CUL3a), CULLIN3b (CUL3b), CULLIN4 (CUL4), and ANAPHASE PROMOTING COMPLEX 2 (APC2) (Choi *et al.*, 2014). Available findings so far suggest that CRL-type E3 complexes, such as APC/C (Bao *et al.*, 2013), SCF^{COI1} (Zhang XC *et al.*, 2015), SCF^{CPR1} (Marino *et al.*, 2012), SCF^{ACIF1} (van den Burg *et al.*, 2008), CRL3 (also denoted as BC3B for BTB/Cullin3/BTB) (Fu *et al.*, 2012) and $CUL4^{DDB1}$ (Liu *et al.*, 2012a), are intimately involved in plant immunity.

The paralogues of the transcription cofactor NONEXPRESSOR OF PR GENES 1 (NPR1), NPR3 and NPR4, contain the BTB domain and have been shown to function as adaptors of the CUL3 ubiquitin E3 ubiquitin ligase to mediate NPR1 degradation in an SA-regulated manner (Fu *et al.*, 2012). CUL3 ubiquitin ligase is also involved in calcium ion-mediated plant immunity. It is well known that calcium influx is one of the early events critical for the activation of defence responses. The *Arabidopsis thaliana* Ca^{2+}/calmodulin-binding transcription factor, SIGNAL RESPONSIVE1 (AtSR1, also known as CAMTA3), has been shown to be a negative regulator of plant immunity and to repress the expression of ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), a well-known regulator of the SA level (Du L *et al.*, 2009). The AtSR1 INTERACTION PROTEIN 1 (SR1IP1) is a CUL3-based E3 ubiquitin ligase that positively regulates plant immunity by degradation of the defence suppressor AtSR1 (Zhang *et al.*, 2014). Consistently, the loss-of-function mutant of SR1IP1 is more susceptible, whereas overexpression of SR1IP1 confers enhanced resistance to bacterial pathogens (Zhang *et al.*, 2014).

CUL4 has been shown to assemble with UV-DAMAGED DNA BINDING PROTEIN 1 (DDB1, also called HIGH PIGMENT-1, HP1) to form CUL4–DDB1-based ubiquitin ligase (Liu *et al.*, 2012a). CUL4–DDB1-based ubiquitin ligase has been implicated in tomato resistance to non-tumorigenic *Agrobacterium tumefaciens* (Liu *et al.*, 2012a). Exogenous SA-triggered induction of *SlPR1a1* and several PTI marker genes and enhanced resistance to *Agrobacterium* are compromised in the *hp1* (*ddb1*) mutant, suggesting that CUL4–DDB1-based E3 may act through interaction with the SA-mediated PTI pathway (Liu *et al.*, 2012a). The Arabidopsis chaperone heat shock protein 90 (HSP90) has been shown previously to interact with RAR1 and SUPPRESSOR OF THE G2 ALLELE OF SKP1 VARIANT 1 (SGT1), which is required for NLR immune receptor/R protein RPS2-mediated disease resistance (Takahashi *et al.*, 2003). Plant CRL E3 ubiquitin ligase SCF^{CPR1}, which contains the F-box protein CONSTITUTIVE EXPRESSOR OF PR GENES 1 (CPR1), negatively regulates the stability of the NLR immune receptors SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1 (SNC1) and RPS2 (Cheng *et al.*, 2011). SNC1 is also subjected to negative regulation by the SUPPRESSOR OF rps4-RLD1 (SRFR1), which interacts with

an isoform of SGT1, SGT1b (Li *et al.*, 2010). Interestingly, mutations in *HSP90.2* and *HSP90.3* result in heightened accumulation of NLR immune receptors SNC1, RPS2 and RPS4 (Huang *et al.*, 2014). It is thus believed that HSP90 isoforms work with SGT1 and SRFR1 in promoting the formation of SCF^{CPR1} E3 ubiquitin ligase which ubiquitinates immune receptors for degradation. In mammals, tumour necrosis factor receptor (TNFR)-associated factors (TRAFs) are cytosolic RING-type E3 ubiquitin ligases which also act as adaptor proteins that are employed by immune receptors, including Toll-like receptors (TLRs), NLRs and T-cell receptors, for downstream signalling (Napetschnig and Wu, 2013; Xie, 2013). Recently, two Arabidopsis TRAF domain-containing proteins, MUTANT, snc1-ENHANCING 13 (MUSE13) and MUSE14, have been found to be associated with the SCF^{CPR1} E3 ubiquitin ligase, and the loss of both MUSE13 and MUSE14 results in enhanced resistance to pathogen *Pst* DC3000 (AvrRpt2) and NLR (SNC1 and RPS2) accumulation, which leads to the speculation that MUSE13 and MUSE14 work together with the SCF^{CPR1} E3 ubiquitin ligase to modulate the ubiquitination and subsequent degradation of NLRs (Huang *et al.*, 2016). Taken together, these findings highlight the important role of SCF^{CPR1} and its chaperones/adaptors in the modulation of the stability of NLR immune receptors.

In addition to SA signalling and NLR immune receptor-mediated immunity, CRL E3 ubiquitin ligase is also involved in JA signalling which has been shown to regulate plant immunity (Truman *et al.*, 2007). SGT1b and its homologue SGT1a are involved in the maintenance of a steady state level of the F-box proteins COI1 and TIR1, receptors for JA and auxin, respectively. Mutation of SGT1b impairs plant responses to the phytohormones JA, auxin and gibberellic acid, but not brassinolide and ABA in Arabidopsis (Zhang XC *et al.*, 2015). COI1 is an integral part of the Skp1-Cullin-F-box-type E3 ubiquitin ligase, SCF^{COI1} and also a client protein of SGT1b–HSP70–HSP90 chaperone complexes (Xie *et al.*, 1998; Xu *et al.*, 2002; Zhang XC *et al.*, 2015). SCF^{COI1}-mediated ubiquitination and degradation of the negative regulators of JA-responsive transcription factors, JAZ proteins, via the 26S proteasome lead to the rapid activation of JA-mediated responses, including plant immune responses.

The stability of plant CRL E3 ubiquitin ligases themselves is positively regulated through the conjugation of the ubiquitin-like peptide RELATED TO UBIQUITIN (RUB)/NEURAL PRECURSOR CELL EXPRESSED, DEVELOPMENTALLY DOWN-REGULATED 8 (NEDD8) to cullins. RUB modification is antagonized by the CONSTITUTIVELY PHOTOMORPHOGENIC9 (COP9) signalosome (CSN), an evolutionarily conserved eight-subunit complex that cleaves RUB from cullins (Stuttmann *et al.*, 2009). The CSN serves as an activator of CRLs and prevents autocatalytic degradation of several CRL substrate adaptors (Stuttmann *et al.*, 2009). CRL-type E3 ubiquitin ligases known to interact with the CSN include SCF^{TIR1} (Gray *et al.*, 2001) and SCF^{COI1}, which are crucial for auxin and JA signalling, respectively (Feng *et al.*, 2003). The CSN displays a profound effect on JA-dependent plant defence responses and positively regulates plant resistance against herbivorous *Manduca sexta* larvae and the necrotrophic fungal pathogen *Botrytis cinerea* (Hind *et al.*, 2011).

REGULATION OF PLANT IMMUNITY BY UNCONVENTIONAL UBIQUITINATION

Quantitative proteomics analysis of the Arabidopsis ubiquitome has indicated that the abundance of substrate protein-attached ubiquitin chains in terms of the lysine residue linkage of the ubiquitin moieties is in the order K48 > K63 > K11, followed by K33, K6 and K29 linkages at much lower abundance (Kim *et al.*, 2013; Maor *et al.*, 2007). The ubiquitin linkage which could not be detected in Arabidopsis is via K27, which, interestingly, is the only non-surface-exposed lysine of the ubiquitin protein. In addition, the linear ubiquitin chain has also not been identified in the plant ubiquitome (Callis, 2014; Kim *et al.*, 2013).

In mammalian cells, unconventional, non-degradative ubiquitination is vital for the activation, signal transduction and development of the end responses of host innate and adaptive immunity (Jiang and Chen, 2012; Li *et al.*, 2016). It has now become evident that K63-linked polyubiquitination plays an essential role in RETINOIC ACID-INDUCIBLE GENE I (RIG-I)-like receptor (RLR)- and TLR-mediated immune signalling (Jiang and Chen, 2012). Findings to date have indicated that TRAF6-mediated K63 autoubiquitination, as well as K27- and K29-linked and linear ubiquitination of the NF-κβ ESSENTIAL MODULATOR (NEMO, also termed IKKγ), are critical for host antiviral and proinflammatory responses (Davis and Gack, 2015). Linear ubiquitination has also been implicated in the initiation and maintenance of immune signalling on activation by various stimuli and in the prevention of tumour necrosis factor (TNF)-induced cell death (Shimizu *et al.*, 2015). In addition, monoubiquitination and K63-linked ubiquitination have been shown to regulate internalization and endocytotic trafficking of membrane-localized receptors (Haglund and Dikic, 2012; Mukhopadhyay and Riezman, 2007). In contrast with its extensive study in human and animal immune systems, an understanding of the role and underlying mechanistic basis of unconventional ubiquitination in plant innate immunity is still at its infant stage. To date, only K63- and K11-linked ubiquitination and monoubiquitination have been implicated in plant immunity, with the underlying molecular mechanism remaining largely elusive (Fig. 3).

K63-LINKED UBIQUITINATION

It is now known that the E2 enzymes are the major determinants of the topology of polyubiquitin chain (ubiquitin linkage) formation, whereas E3s mainly govern the substrate specificity of the ubiquitination reaction. Of all the E2 enzymes, UBC13

(also called UBE2N in mammals) and its homologues are the only known E2 enzymes that catalyse exclusively the formation of K63-linked ubiquitin chains. In humans and animals, UBC13 heterodimerizes with the cofactor, UBIQUITIN-CONJUGATING ENZYME VARIANT1a (UEV1a). The heterodimer then works with the RING-type E3 ubiquitin ligases TRAF6 and TRAF3 to direct K63-linked ubiquitination of the E3 ubiquitin ligases themselves and various key components of the host immune system. Such K63-linked ubiquitination has been found to be essential to RLR- and TLR-mediated activation of the NF-κB pathway (Jiang and Chen, 2012).

Despite being the second most abundant type of ubiquitination in plants, few plant proteins being modified by K63-linked ubiquitination have been identified. To date, only the PIN-FORMED2 (PIN2) auxin efflux carrier (Leitner *et al.*, 2012) and the steroid hormone receptor kinase BRASSINOSTEROID-INSENSITIVE1 (BRI1) (Martins *et al.*, 2015) have been identified to be substrates of K63-linked ubiquitination. Nevertheless, the involvement of K63-linked polyubiquitination in plant immunity is emerging. In tomato, the intracellular serine/threonine (Ser/Thr) protein kinase Pto acts in conjunction with the NLR immune receptor/R protein Prf to confer resistance to the bacterial pathogen *Pst*. Pto recognizes the *Pst* effector protein AvrPtoB, the C-terminus of which encodes a RING/U-box-type E3 ubiquitin ligase to initiate defence signalling. *Pto* belongs to a small gene family and the Fen kinase encoded by another member of the family does not detect AvrPtoB, but recognizes E3 function-deficient variants of AvrPtoB to induce Prf-dependent HR, which eventually arrests the growth of pathogens at the site of infection (Abramovitch *et al.*, 2003; Rosebrock *et al.*, 2007). Conversely, AvrPtoB interferes with Fen-mediated immunity by using its C-terminal E3 ubiquitin ligase activity to ubiquitinate Fen, which leads to the degradation of Fen by the 26S proteasome (Rosebrock *et al.*, 2007). To further understand the molecular basis of Fen-mediated immunity, tomato Fen-interacting (FNI) proteins were identified. One of the FNI proteins, tomato FEN-INTERACTING PROTEIN 3 (FNI3), was found to encode a Ubc13-type ubiquitin E2 enzyme and to work with its cofactor *Solanum lycopersicum* UEV VARIANT (SUV) to direct K63-linked ubiquitination (Mural *et al.*, 2013). Decreased expression of *Fni3* and another tomato *Ubc13* homologue *Sl-Ubc13-2* or *Suv* in *N. benthamiana* leaves diminished PCD associated with Fen-mediated immunity and PCD elicited by several other resistance proteins and their cognate effectors, indicating that FNI3 and SUV positively regulate plant immunity (Mural *et al.*, 2013). It is unknown at present how FNI3/SUV-directed K63-linked ubiquitination regulates plant immunity. The identification and characterization of the cognate E3 ubiquitin ligase(s) and potential substrate protein(s) for FNI3/SUV-directed K63-linked ubiquitination that are relevant to plant immunity would help to address this question.

BRI1 has been shown recently to be modified by K63-linked polyubiquitin chains *in vivo* (Martins *et al.*, 2015). K63-linked polyubiquitin chain formation functions as a dual targeting signal for BRI1 internalization and sorting along the endocytic pathway, highlighting the role of K63-linked ubiquitination in the regulation of brassinosteroid (BR) signalling (Martins *et al.*, 2015). BR signals, which control many aspects of plant growth, development and immunity, are perceived at the cell surface by the plasma membrane-localized receptor complex composed of the receptor kinase BRI1 and its co-receptor, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1). The perception of BRs results in inactivity of a negative regulator, BR-INSENSITIVE 2 (BIN2), in BR signalling and the accumulation of dephosphorylated BRI1-EMS-SUPPRESSOR 1 (BES1) in the nucleus, which acts as an active transcription factor that affects the expression of various BR signalling effector genes (Shin *et al.*, 2016). In addition to BRI1, BAK1 also forms a receptor complex with the immune receptor FLS2 to modulate early events in flagellin-triggered plant PTI (Chinchilla *et al.*, 2007). These findings together raise the possibility that BR signalling and plant immunity are interrelated in plants. Indeed, activation of BR signalling inhibits FLS2-mediated PTI and the inhibition is downstream or independent of the FLS2–BAK1 complex (Albrecht *et al.*, 2012). In addition, overexpression of BRI1 specifically enhances plant susceptibility to hemibiotrophic pathogens (Belkhadir *et al.*, 2012), whereas the *bri1* mutant shows increased disease resistance against necrotrophic and hemibiotrophic pathogens (Goddard *et al.*, 2014), demonstrating that BR signalling functions antagonistically with disease resistance against a broad range of pathogens. Considering that K63-linked ubiquitination of BRI1 plays a negative role in BR signalling (Martins *et al.*, 2015) and that BR signalling antagonizes PTI signalling, it is not illogical to deduce that K63-linked ubiquitination of BRI1 probably has a positive impact on plant immunity, which is in line with the finding that FNI3- and SUV-directed K63-linked ubiquitination positively regulates plant immunity (Mural *et al.*, 2013). Future research on the relationship of K63-linked polyubiquitination of BRI1 to plant immunity and the identification of the cognate E3 ubiquitin ligase that catalyses the K63-linked ubiquitination of BRI1 will probably shed light on how K63-linked ubiquitination regulates plant immunity and the cross-talk between BR and plant immune signalling.

K11-LINKED UBIQUITINATION

In addition to conventional K48-linked ubiquitination, modification of substrate proteins by polybubiquitin chains with Lys-11 (K11) linkage also signals substrates for 26S proteasome-dependent degradation in mammalian cells (Min *et al.*, 2015). In humans and animals, the CRL-type E3 ubiquitin ligase APC/C works with the ubiquitin E2 enzyme UBE2S to assemble K11-linked ubiquitination in cells that are released from mitotic arrest, and the K11-linked

ubiquitination-mediated degradation of anaphase substrates is required for cells to progress into mitotic exit (Min *et al.*, 2015). Recently, the Arabidopsis homologue of UBE2S, UBC22, has been revealed to be required for female gametophyte development and to catalyse the formation of K11-linked ubiquitination *in vitro* (Wang *et al.*, 2016), suggesting that the function of UBE2S and UBC22 is biochemically conserved in directing K11-linked ubiquitination. In Arabidopsis, APC/C regulates the progression of cell cycles (Bao *et al.*, 2013). Meanwhile, overexpression of the negative regulators of APC/C, OMISSION OF THE SECOND DIVISION 1 (OSD1) and its homologue UV-B-INSENSITIVE 4 (UVI4), or reduction of the function of an APC complex subunit, APC10, resulted in enhanced host immunity (Bao *et al.*, 2013). These findings suggest a link between cell cycle progression and plant immunity, and an indirect connection of K11-linked ubiquitination to the alteration of plant immunity.

During the co-evolution of pathogens and plants, pathogens have developed diverse effector proteins that are secreted into host cells to subvert plant immunity (Banfield, 2015). One of the effectors, XopL, secreted by the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria*, exhibits C-terminal E3 ubiquitin ligase activity *in vitro* and *in planta*, and suppresses plant defence in an E3 ubiquitin ligase activity-dependent manner (Singer *et al.*, 2013). XopL specifically interacts with plant ubiquitin E2 enzymes AtUBC28 and AtUBC11 to mediate the formation of predominantly K11-linked polyubiquitin chains (Singer *et al.*, 2013). It has been proposed that XopL directs K11-linked ubiquitination of plant proteins for immunity suppression, but the host substrates of the modification remain to be identified.

MONOUBIQUITINATION

Like K63-linked ubiquitination, modification by monoubiquitination usually leads to a non-degradative fate for the substrate proteins in which their activity and subcellular localization may be affected (Hicke, 2001). In humans and animals, monoubiquitination and multiubiquitination are well known to regulate DNA repair, endocytosis and gene expression (Hicke, 2001; Sadowski *et al.*, 2012). In plants, monoubiquitination has been shown to be involved in multiple cellular and physiological processes, including protein endocytosis (Barberon *et al.*, 2011), chromatin remodelling (Liu *et al.*, 2007; Menard *et al.*, 2014), development (Bratzel *et al.*, 2010; Cao *et al.*, 2015), flowering time (Cao *et al.*, 2008a) and immunity (Dhawan *et al.*, 2009; Zhang Y *et al.*, 2015; Zou *et al.*, 2014). The Arabidopsis ubiquitin E2 enzymes AtUBC1/AtUBC2 and RING-type E3 ubiquitin ligases HUB1/HUB2 together mediate monoubiquitination of histone H2B, which is involved in growth and development (Xu L *et al.*, 2009). HUB1 also works with MED21, a subunit of the Arabidopsis mediator complex that regulates RNA polymerase II, to regulate host defence against necrotrophic pathogens, suggesting a role of monoubiquitination of histone H2B in plant immunity (Dhawan *et al.*, 2009). A recent study has indicated that monoubiquitination of H2B at the R gene *SNC1* locus affects the expression of *SNC1*, which thus affects the immune responses in plants. Moreover, H2B monoubiquitination at *SNC1* is enhanced by pathogen infection (Zou *et al.*, 2014). The study therefore presents a direct link of H2B monoubiquitination to plant immunity. More recently, tomato HUB1 and HUB2, SlHUB1 and SlHUB2, have been found to contribute to resistance against *B. cinerea*, most likely through the modulation of the balance between SA- and JA/ethylene-mediated signalling pathways (Zhang Y *et al.*, 2015). These findings together implicate H2B monoubiquitination in the tuning of plant immunity, probably via an effect on the expression of plant immunity-associated genes. However, direct modification of other plant substrate proteins, such as signalling components of the plant immune system by monoubiquitination, have not yet been reported.

CONCLUSION AND OUTLOOK

It is now well accepted that plants possess a sophisticated innate immune system that is tightly regulated and often fine tuned, which enables timely and efficient plant immune responses to ward off infection by various pathogens. Of the many key regulatory mechanisms discovered in the past decade, the breadth and depth of the involvement of ubiquitination in the plant immune network has been dominant, particularly for the E3 ubiquitin ligases from different subfamilies. Emerging evidence so far indicates that ubiquitination plays essential roles in the regulation of the abundance, activity and subcellular localization of plant immunity-associated proteins, and their interactions with other cellular molecules. In several cases, such as rice SPL11 and Arabidopsis PUB12/13, independent studies on different pathosystems have converged to the same E3 enzyme (orthologues), indicating an essential and conserved role for the E3 enzymes among different plant species. Despite these advances, however, a mechanistic understanding of the involvement in the plant innate immune network has not been established for the majority of the E3 enzymes listed in Table 1. The identification and characterization of the cognate substrate proteins of these E3 enzymes in plant immunity will thus continue to be of importance. In addition, in contrast with the E3 enzymes, very few investigations have been reported that implicate ubiquitin E1 and E2 enzymes in plant innate immunity, although ubiquitin E2 enzymes are now known to play a major role in determining the topology/linkage of polyubiquitin chain formation. To date, a very limited understanding of unconventional ubiquitination in plants has been achieved, despite the fact that, in human and animals, ubiquitination of key regulators and signalling components by unconventional, non-degradative ubiquitination, such as K63-linked ubiquitination, has become a central theme of innate and adaptive immunity. As such, this knowledge gap remains to be filled for certain key plant immune

processes, such as endocytosis and intracellular trafficking of the pattern recognition receptors (PRRs) that mediate PTI, which are probably regulated by monoubiquitination and/or K63-linked ubiquitination. In this regard, in-depth characterization at the genome scale of plant E2 enzymes and the pinpointing and characterization of the plant E2–E3 network which catalyses unconventional ubiquitination by enabling tools, such as quantitative proteomics and genome editing, will be the first steps to address this question, and would certainly also shed light on the unconventional ubiquitination in plant biology in general.

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