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REVIEW PAPER

E3 ubiquitin ligases and plant innate immunity

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Abstract

In yeast and in animals the ubiquitin–proteasome system (UPS) is responsible for removing or modifying most abnormal peptides and also short-lived cellular regulators. The UPS therefore influences many processes such as the cell cycle, signal transduction, transcription, and stress responses including defence. In recent years, similar regulatory roles have been identified in plants. In *Arabidopsis*, mutations in the ubiquitin–proteasome pathway block development, circadian rhythms, photomorphogenesis, floral homeosis, hormone responses, senescence, and pathogen invasion. Plants have evolved an armoury of defence mechanisms that allow them to counter infection. These encompass both basal responses, triggered by recognition of conserved pathogen-associated molecular patterns, and pathogen-specific responses, mediated via pathogen- and plant-specific gene-for-gene recognition events. The role of E3 ubiquitin ligases in mediating plant defence signalling is reviewed and examples where pathogens impinge on the host's ubiquitination machinery acting as molecular mimics to undermine defence are also highlighted.

Key words: Disease, plant, resistance, ubiquitin.

Plant immunity

Plant pathogenic microbes fall into two categories which derive nutrients either from dead or dying cells (necrotrophs) or from living host tissues (biotrophs). Biotrophic plant pathogens use diverse life strategies. Pathogenic bacteria proliferate in the plant apoplast after entering through existing wounds, stomata, or hydathodes (Glazebrook, 2005). From the apoplast, bacterial pathogens access the plant cell through a secretion pilus (Glazebrook, 2005). Pathogenic and symbiotic fungi and oomycetes can invaginate feeding structures (haustoria) into the host cell plasma membrane (Glazebrook, 2005). Early pathogen perception events occur at the extracellular matrix and host cell plasma membrane where the outcome of the interaction is determined (Jones and Dangl, 2006). To influence host defence responses and enhance microbial fitness, these diverse pathogen classes all deliver effector molecules (avirulence factors) into the plant cell (Dangl and McDowell, 2006).

Current research suggests that the inducible plant immune system can be broadly divided into two branches (Jones and Dangl, 2006). One of these mediates the perception of microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs, respectively) such as flagellin through transmembrane pattern recognition receptors (PRRs) (Schwessinger and Zipfel, 2008). Defence responses activated by PAMPs are collectively termed PAMP-triggered immunity (PTI) or basal resistance (Schwessinger and Zipfel, 2008). The second branch acts primarily inside the cell using disease resistance (R) proteins which recognize pathogen-delivered effectors or their effects on host proteins. R protein-mediated defences are termed effector-triggered immunity (ETI) or gene-for-gene resistance (Jones and Dangl, 2006) (Fig. 1).

Activation of PTI by PAMP recognition is proposed to be the plant's first inducible response to microbial perception (Schwessinger and Zipfel, 2008). In the majority of cases, PTI halts pathogen growth at an early infection stage due to the induction of pathogen-responsive genes, production of reactive oxygen species, and deposition of callose

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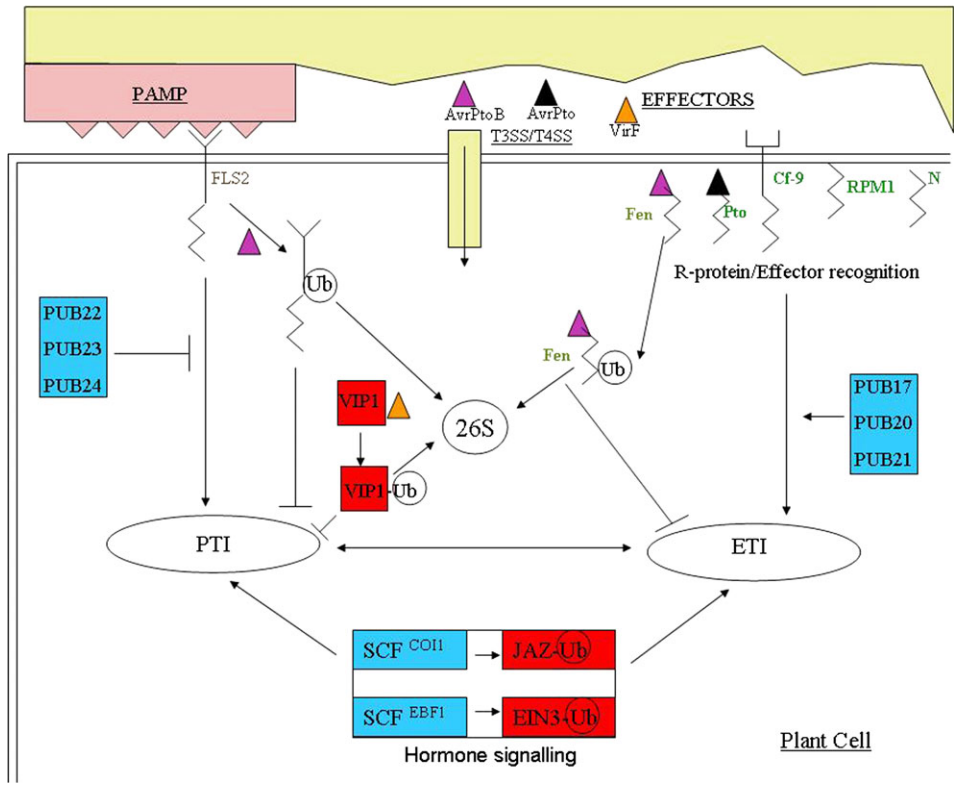

Fig. 1. An overview of plant–pathogen interaction leading to immune responses and the effect of a selection of E3 ubiquitin ligases in defence signalling. For more details, please refer to the text. The pathogen is coloured yellow; PAMPs are pink; effector molecules are triangular; host signalling proteins are red; PAMP receptors are black; effector receptors or R proteins are green; and plant E3 ligases are coloured blue. Hormone signalling is boxed for simplification. T3SS, type III secretion system; T4SS, type IV secretion system, Ub, ubiquitin; PTI, PAMP-triggered immunity; ETI, effector-triggered immunity.

to reinforce the cell wall at sites of infection (Schwessinger and Zipfel, 2008).

Biotrophic pathogens deploy effector proteins which disrupt plant immune responses and promote successful infection. Direct or indirect recognition of effectors by R proteins initiates ETI, which is an amplified and accelerated PTI response resulting in disease resistance (Jones and Dangl, 2006). ETI is usually accompanied by a localized hypersensitive cell death response (HR) at the infection site. R proteins have been classified into five distinct classes (Jones and Dangl, 2006), most of which contain characteristic leucine-rich repeat (LRR) domains. LRR domains are detected in diverse proteins and function as sites of protein–protein interaction, peptide–ligand binding, and protein–carbohydrate interaction (Kajava, 1998). Comparative sequence analysis indicates that R protein specificity results primarily from hypervariability in the LRR region (Dangl and Jones, 2001). R proteins mediate perception of effectors from diverse kingdoms and integrate recognition of bacterial, viral, fungal, and oomycete pathogens to activate similar downstream defence responses which result in disease resistance (Dangl and Jones, 2001).

In the majority of cases, ETI triggered during gene-for-gene resistance is proposed to be most accurately described by the 'guard hypothesis' (Dangl and Jones, 2001). In the guard hypothesis, R proteins are proposed to monitor the integrity of host effector targets (Dangl and Jones, 2001). Alteration of host targets by pathogen-derived effectors is perceived by specific R proteins, leading to the activation of ETI (Jones and Dangl, 2006).

PAMP-triggered immunity (PTI)

Perception of conserved microbe structural components termed PAMPs leads to the prompt activation of plant defences through PTI. PTI signalling in plants has been most extensively characterized in the case of the flagellin, which is an archetypal PAMP and triggers defence responses in various plants (Schwessinger and Zipfel, 2008). Flagellin subunits collectively form the bacterial flagellum required for motility and virulence, and distinct conserved flagellin domains are recognized by mammalian and plant receptors TLR5 and FLS2, respectively (Zipfel and Felix, 2005). *Arabidopsis* FLS2 (FLAGELLIN-SENSING2) is a LRR receptor kinase which directly binds the 22 amino acid flagellin epitope flg22 (Zipfel and Felix, 2005), and *fls2* mutants exhibit enhanced susceptibility to bacterial infection (Zipfel *et al.*, 2004). Characterization of other flg22-insensitive mutants led to the elucidation of the mitogen-activated protein kinase (MAPK) cascade and WRKY signalling pathways that function downstream of flagellin

perception (Asai *et al.*, 2002). Similar signalling responses have been reported during the perception of bacterial elongation factor Tu (EF-Tu) by the LRR receptor kinase EFR, and the elicitation of PTI in *fls2 efr-1* double mutants indicates the existence of other PAMP receptors in *Arabidopsis* (Zipfel *et al.*, 2006). Molecules with PAMP activity have also been identified in fungal and oomycete plant pathogens (Nurnberger *et al.*, 2004).

Effector-triggered immunity (ETI)

Beyond the amplified induction of PTI responses, activation of ETI by pathogen effectors results in rapid production of reactive oxygen intermediates (ROIs), termed the oxidative burst, and development of localized programmed cell death known as the HR (Nimchuk *et al.*, 2003). ETI activation causes elevated salicylic acid (SA) accumulation which induces transcription of various pathogenesis-related (PR) genes and the activation of systemic acquired resistance (SAR) (Durrant and Dong, 2004). The oxidative burst is proposed to serve a direct antimicrobial effect and also initiates signal activation for other downstream defence responses (discussed below), whilst the HR is thought to act to suppress biotroph infection by restricting pathogen access to water and nutrients (Nimchuk *et al.*, 2003). The activation of common disease resistance signalling pathways results from the perception of bacterial, viral, fungal, oomycete, and nematode pathogen effectors by their associated R proteins (Dangl and Jones, 2001). Despite the broad taxonomic origins of known plant pathogens and the presumed diversity in their effector molecules, only five structural classes of R protein have been reported, with the presence of LRR domains being a recurring theme in the majority of cases (Dangl and Jones, 2001).

Signal transduction during PTI

Basal resistance (PTI) triggered by PAMP perception represents the frontline of inducible defence and triggers diverse signalling responses. These include the rapid changes in intracellular Ca^{2+} flux, induction of an oxidative burst, transcriptional reprogramming, cell wall reinforcement, and receptor endocytosis (Altenbach and Robatzek, 2007; Schwessinger and Zipfel, 2008). PAMP perception results in SA accumulation, and recent reports indicate that disruption of SA biosynthesis in the *Arabidopsis sid2* mutant results in compromised PTI defences against virulent *Pseudomonas syringae* (Tsuda *et al.*, 2008). PTI induction also results in the activation of MAPK kinase cascades, and the *Arabidopsis* MKK1–MPK3/MPK6 kinase module has been shown to act downstream of the flagellin receptor FLS2, leading to the activation of WRKY22/29 transcriptional targets (Asai *et al.*, 2002). Microarray analysis indicates that PAMP perception induces rapid changes in gene expression, with a significant expression overlap during PTI induced by fungal or bacterial PAMPs (Zipfel *et al.*, 2006). Significant overlap has also been reported between PTI and ETI transcriptomes, underscoring the fact that ETI includes amplified aspects of the PTI response (Zipfel *et al.*, 2006).

Signal transduction during ETI

Gene-for-gene resistance or ETI is superimposed onto basal resistance mechanisms and is characterized by a sustained burst of ROIs, induction of localized cell death (HR) with activation of defence gene expression and resistance in systemic tissues (SAR) (Jones and Dangl, 2006). Key proteins that regulate ETI have been identified in *Arabidopsis*, with isolated mutants indicating that R protein activation leads to activation of the oxidative burst, causing a change in cellular redox status which induces HR and SA accumulation (Nimchuk *et al.*, 2003). Elevated SA levels potentiate the HR and lead to the induction of defence genes and the subsequent development of SAR (Nimchuk *et al.*, 2003). Signal transduction events which cause disease resistance following R protein activation during ETI occur through multiple interacting pathways which are regulated by increased transmembrane ion flux (Ca^{2+}, K^+, and H^+), nitric oxide production, and increased SA accumulation, amongst many other factors (Hofius *et al.*, 2007).

Genetic screens for loss of resistance to *Peronospora parasitica* and *Pseudomonas syringae* identified NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE) and EDS1 (ENHANCED DISEASE SUSCEPTIBILITY) as components required to mediate signalling by distinct *R* genes (Aarts *et al.*, 1998). It is now established that NDR1 is required for resistance mediated by most CC-NB-LRR *R* genes whilst EDS1 mediates resistance signalling through the TIR-NB-LRR R gene class (Dangl and Jones, 2001).

Following activation of ETI, local SA levels are dramatically increased through the isochorismate synthesis pathway, and regulate HR development and downstream signalling events which induce defence gene expression leading to SAR (Durrant and Dong, 2004). SA is proposed to function through feedback loops both upstream and downstream of the HR, establishing an SA-dependent gradient which restricts cell death development to the initial infection site (Hofius *et al.*, 2007).

HR development is also subject to positive and negative feedback regulation through the interacting effects between SA, ROIs, ET (ethylene) and JA (jasmonate). Together, SA and ROIs are proposed to trigger cell death initiation, causing an increase in ET which stimulates further ROS production and SA synthesis in surrounding cells to effect cell death propagation (Hofius *et al.*, 2007). JA has been reported to exert both inhibitory and pro-cell death regulation through perception of distinct ROI species, but is proposed to function primarily through antagonistic effects on ET signalling to promote lesion containment (Hofius *et al.*, 2007).

The ubiquitin–26S proteasome system

All aspects of plant physiology and development are controlled by regulated synthesis of new polypeptides and degradation of existing proteins. Within this 'protein cycle', the intricate transcriptional and translational events leading to protein synthesis are relatively well characterized. Studies conducted in the last decade have greatly improved our appreciation of the corresponding catabolic processes that regulate protein degradation. Protein degradation serves key housekeeping functions by removing misfolded proteins and in the maintenance of free amino acids during growth and starvation. It is also essential for many aspects of cellular regulation by removing rate-limiting enzymes and suppressing regulatory networks to fine-tune homeostasis and adapt to new environments.

Current data indicate that the ubiquitin (Ub)–26S proteasome pathway functions as the principle proteolytic system in eukaryotes and is extensively involved in plant cellular signalling. In this pathway, Ub serves as a reusable tag (Fig. 2) which directs target proteins for selective turnover. Polymeric chains of Ub are covalently attached to protein targets through the iterative action of a three-step (E1 > E2 > E3) conjugation cascade. Resulting ubiquitinated target proteins are directed to the 26S proteasome for degradation with the concomitant release of Ub moieties for reuse.

The ubiquitin protein

Ub is a 76 amino acid globular protein found in all eukaryotes; its sequence is highly conserved and only two residues differ between yeast and human species (Callis *et al.*, 1995) (Fig. 2). It is the prototypical member of the ubiquitin-like (Ubl) protein family which covalently modify target proteins to alter various aspects of their regulation (Jentsch and Pyrowolakis, 2000). Ub assumes a compact structure with a five-strand mixed β-sheet forming a cavity into which a single α-helix fits diagonally to form a characteristic 'Ub fold'. Numerous intramolecular hydrogen bonds impart Ub with high stability, presumably to encourage recycling rather than proteolysis during the conjugation/degradation process. The flexible C-terminus of Ub protrudes from the Ub fold and terminates with an essential glycine residue. The carboxyl group of this glycine functions as an initiation site for the covalent attachment of Ub to substrates.

Ub gene family members (UBQs) are detected either as Ub polymers in which multiples (typically 4–6 in *Arabidopsis*) of the 228 bp coding region are concatenated head to tail or as one of three different fusion proteins (Callis *et al.*, 1995). The Ub fusion genes encode either one of two different ribosomal subunits or the Ubl RUB-1 (related to ubiquitin) protein fused to the C-terminus of Ub (Callis *et al.*, 1990). In all cases Ub fusion precursors are cleaved at the terminal glycine by deubiquitinating enzymes (DUBs) to release active monomers (Amerik and Hochstrasser, 2004).

Ub contains seven lysines (K6, K11, K27, K29, K31, K48, and K63). To target substrates for degradation by the proteasome, covalent inter-Ub linkages are made from the C-terminal glycine to the K48 of the previous Ub moiety (i.e. the G76–K48 isopeptide bond) to form Ub chains (poly-Ub) (Fushman and Pickart, 2004). Poly-Ub chains of at least four Ub moieties (tetra-Ub) are required to provide an efficient proteasome delivery signal (Thrower *et al.*, 2000).

The ubiquitin conjugation cascade

Attachment of free Ub moieties to appropriate substrates proceeds by an ATP-dependent E1 > E2 > E3 enzyme conjugation cascade (Table 1). The cascade starts with E1 (or Ub-activating enzyme). The E1 enzyme catalyses the formation of an acyl phosphoanhydride bond between the adenosine monophosphate (AMP) of ATP and the C-terminal glycine carboxyl group of Ub. Activated Ub then forms a stable intermediate by binding directly to an E1 cysteine via a thiolester linkage. This activated Ub is transferred from E1 to E2 (or Ub-conjugating enzyme) by transesterification. The E2–Ub intermediate delivers Ub onto a substrate acceptor lysine using an E3 (or Ub ligase). E3 enzymes impart substrate recognition to the process and either promote direct transfer of Ub to substrates from E2 or form a final E3–Ub intermediate prior to transfer. The end-product is an Ub–protein conjugate containing an isopeptide bond between the C-terminal glycine of Ub and the lysyl ϵ-amino group in the substrate.

After attachment of an initial Ub moiety to a substrate, additional Ubs are ligated to specific internal lysine residues on the first Ub to form poly-Ub chains. Whether Ub chains are extended by ligation of pre-assembled poly-Ub or by iterative rounds of E3-based ligation is currently unclear. Whilst linkages through all seven Ub lysines have been

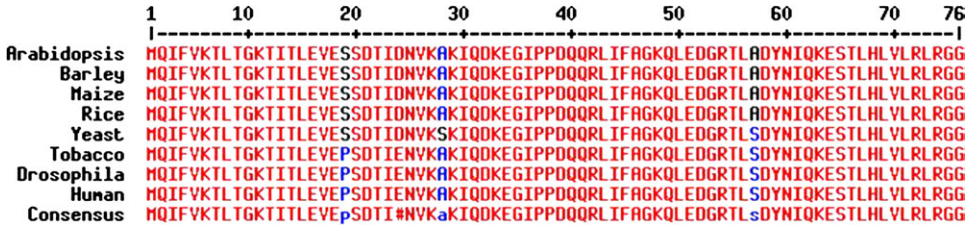

Fig. 2. Amino acid alignment of ubiquitin from various model organisms. The figure shows the high conservation of amino acids in the ubiquitin protein. Numbers indicate amino acid positions. Red indicates sequence identity while blue represents similarity with respect to consensus sequences derived from the Multalin program (Corpet, 1988).

detected *in vivo*, poly-Ub chains linked through lysine 48 (K48) predominate in the cell and present a proteasome targeting/recognition signal (Fushman and Pickart, 2004). Upon delivery to the proteasome, ubiquitinated substrates have poly-Ub chains removed by DUBs prior to unfolding, import, and proteolysis (Hartmann-Petersen *et al.*, 2003).

Although Ub was first identified in the context of proteolysis (Hershko and Ciechanover, 1992), it has become increasingly clear that the addition of single Ub moieties (mono-ubiquitination) (Hicke, 2001) or alternative Ub chain linkage configurations can impart diverse consequences on substrates (Fushman and Pickart, 2004). Other than the archetypal K48 linkage, non-proteolytic signalling by K63-linked poly-Ub chains has been shown to mediate DNA repair, trafficking, and kinase activation (Fushman and Pickart, 2004). Whilst chains linked through K29 and K6 have been observed, their precise function is not currently clear (Fushman and Pickart, 2004).

Ubiquitin-activating enzymes

There are three major stages during the tagging of a protein to ubiquitination and consequently there are three major enzyme classes. The first class of enzymes is the ubiquitin-activating or E1 enzymes (115–125 kDa) which catalyse the two-step reaction to activate Ub and require an ATP energy source. A typical E1 enzyme contains a nucleotide-binding site that is enough for the activation of Ub for the entire array of downstream conjugating enzymes (Fushman and Pickart, 2004). The *Arabidopsis* genome encodes two E1 enzymes, of which at least one (AtUBA1) is localized in the nucleus.

Ubiquitin-conjugating enzymes

The next class of enzymes, the ubiquitin-conjugating (UBC) or E2 enzymes can be divided into four major types with each containing a 16 kDa conserved domain (UBC domain) which contains the active cysteine residue for the trans-esterification reaction with Ub-loaded E1. Generally there seem to be more E2 enzymes found in a genome than E1 enzymes (Mazzucotelli, 2006). Currently there are 37 E2 enzymes identified in the *Arabidopsis* genome. Sequence analysis has clustered *Arabidopsis* E2s into 12 distinct subfamilies (Wiborg *et al.*, 2007), but the majority of subtypes currently await functional classification.

Table 1. The different enzyme classes involved in the ubiquitin conjugation cascade and the respective number of genes presently found in the model plant *Arabidopsis*

Enzyme class	Number of genes
E1	2
E2	37
E3	1415

See text for further details.
E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase.

Ubiquitin ligase enzymes

The final class, ubiquitin–protein ligases or E3 enzymes are most abundant. There are about 1415 E3 Ub ligases in *Arabidopsis* (Mazzucotelli, 2006) (Table 2). Generally E3 enzymes allow the interaction between E2 enzymes and the target protein by tethering them in close proximity. There are two mechanisms of transfer of Ub from the E2 enzyme to the target: in the first, the E3 enzyme does not become ubiquitinated instead it simply catalyses the transfer; whilst in the second the ubiquitinated E3 enzyme is an intermediate between transfer to the target. The domains present in the different subgroups determine the mechanistic nature. E3 enzymes that contain the HECT [homologous to E6-associated protein (E6AP) C-terminus] domain operate through the intermediate mechanism, whereas those that contain the RING (really interesting new gene) operate without the intermediate. RING E3 ligases can act independently or as part of a multisubunit complex such as SCF (Skp, cullin, F-box-containing complex). HECT domain E3 ligases have only been found to function as a single component. Multisubunit RING domain E3 ligases are based on a modular design, with substrate recognition components being spatially distinct from Ub-conjugating components. Multicomponent E3 ligases typically contain a catalytic RING module, e.g. RBX1 or APC11, an assembly platform module, e.g. CUL1-4 or APC2, and a substrate recognition module, e.g. F-box, BTB, or DDB1 (reviewed by Haglund, 2005). Recognition modules such as the F-box proteins are highly versatile and mediate protein–protein interactions, giving rise to substrate specificity. Recently, 724 F-box domain proteins (Gagne *et al.*, 2002; Mazzucotelli, 2006) were uncovered in the *Arabidopsis* genome; from these data functional pathways can begin to be deciphered. Higher levels of control can be exerted over the system as other components such as RBX1 and the E2- and CUL1-binding subunits (in the case of SCF E3 ligases) can also differ depending on requirements. Sometimes these

Table 2. *Arabidopsis* E3 ligase components and the number of genes categorized by subclass

E3 component	Number of genes within the subclass
HECT	7
PUB	49
RING	499
ASK	21
CUL	11
F-box	724
BTB	81
CUL4-DDB	5
APC	18

See text for further details.
APC, anaphase-promoting complex; ASK, *Arabidopsis* Skp1-related; BTB, bric a brac, tramtrack, and broad complex; CUL, cullin; CUL4-DDB, cullin4-damaged DNA-binding protein; F-box; cyclin F proteins; HECT, homology to E6-AP C-terminus; PUB, plant U-box; RING, really interesting new gene.

multisubunit complexes have another module in contact with the assembly platform and substrate recognition module for, for example, SKP1, or the ASK (*Arabidopsis* SKP1-related) can be termed as an adaptor in SCF complexes. Multisubunit E3 complexes of modular design greatly increase the number of substrates available whilst the number of gene products required remains relatively low.

The RING domain-related U-box domain (Haglund, 2005) also interacts with E2 enzymes, and U-box proteins are based on a modular design similar to F-box proteins but with the E2 recognition occurring through the U-box and a domain for substrate recognition such as armadillo (ARM) repeat domains at the C-terminus. The U-box domain is similar in structure and function to the RING domain (Haglund, 2005).

The 26S proteasome

The 26S proteasome is a 2 MDa ATP-dependent proteolysis complex which degrades Ub-tagged substrates. Whilst initial characterization of the complex was derived from studies of yeast and mammalian proteasomes, subsequent studies in rice and *Arabidopsis* indicate a similar design (Fu *et al.*, 1998). The 26S proteasome is comprised of 31 subunits divided into two subcomplexes, the 20S core protease (CP) and 19S regulatory particle (RP).

The CP functions as a non-specific ATP- and Ub-independent protease which assumes a cylindrical structure by the assembly of four heptameric rings. The peripheral rings are composed of seven related α-subunits and the central rings are composed of seven related β-subunits in an α1–7 α1–7 β1–7 α1–7 configuration (Wolf and Hilt, 2004). Initial crystallography studies of the CP in yeast reported a large central chamber into which face protease active sites contributed by the β1, β2, and β5 subunits (Wolf and Hilt, 2004). These three proteases generate peptidylglutamyl, trypsin-like, and chymotrypsin-like activities, imparting the capacity to cleave most peptide bonds (Wolf and Hilt, 2004).

The RP associates with either end of the CP and confers ATP dependence and poly-Ub recognition to the proteasome. The RP is composed of 17 subunits which form two subcomplexes termed Lid and Base. The Base sits directly over the CP α-ring channel and comprises a ring of six related AAA-ATPases (RPT1–6) and three non-ATPase subunits (RPN1, 2, and 10). The Lid interacts with the Base via RPN10 (Fu *et al.*, 2001) and contains the remaining non-ATPase subunits (RPN 3, 5–9, and 11–12). The overall structure–function relationships between RP subunits remain to be clarified, but key functions have been ascribed to individual subunits (Hartmann-Petersen *et al.*, 2003). Cooperatively the RP Base and Lid mediate recognition of K48-linked poly-Ub chains, removal of covalently bound Ub moieties, unfolding of targeted substrates, pore gating, and substrate import to the proteasome.

Involvement of E3 ubiquitin ligases in plant defence

Apart from a possible role for *MOS5* (which encodes the nuclear E1 enzyme AtUBA1 in *Arabidopsis*) in PTI and ETI triggered by several avirulent genes (Goritschnig *et al.*, 2007), so far evidence for the role of E1 and E2 enzymes in either ETI or PTI signalling is limited; therefore, this review will focus on the regulatory function of E3 ligases in plant immune signalling for which there is emerging evidence.

Regulation of defence gene expression by the signalling hormones ET and JA has been linked to ubiquitination. The EIN3 (ETHYLENE INSENSITIVE 3) transcription factor family are key components of ET signalling and have been reported to control the transcription of numerous defence-related genes including oxidative burst regulators and a subset of PR genes (Dreher and Callis, 2007). The stability of EIN3-type transcription factors is regulated by Ub SCF E3 ligase complexes containing the F-box subunits EBF1 or EBF2 (EIN3 binding F-box) (Delauré *et al.*, 2008). JA signalling has also been linked to ubiquitination through the identification of the *coi1* mutant in *Arabidopsis*. COI1 is an SCF F-box subunit which is implicated in most JA-mediated signalling responses including defence against herbivores and biotrophic pathogens (Turner *et al.*, 2002) (Fig. 1). Positive and negative regulators of plant defence signalling pathways have been identified in multiple E3 ubiquitin ligase classes resulting from elicitor/avirulence induction studies and genetic screens for pathogenesis-related phenotypes (Dreher and Callis, 2007).

Reported F-box defence regulators include SON1 (SUPRESSOR OF NIM1-1). The *Arabidopsis son1* mutant was identified in the *npr1/nim1* background as a negative defence regulator which is implicated in SAR-independent resistance against virulent *P. parasitica* and *P. syringae* strains (Kim and Delaney, 2002). Tobacco transcript profiling experiments during ETI elicited by the *Cladisporium fulvum* effector Avr9 led to the identification of numerous up-regulated ACRE (Avr9/Cf9 rapidly elicited) genes, several of which encode Ub E3 ligases (Durrant *et al.*, 2000). One such gene is *ACIF1* (Avr9/Cf-9-INDUCED F-BOX 1) which encodes an F-box protein that has been implicated as a positive regulator of HR and resistance mediated by the *R* genes *Cf-9*, *Pto*, and *N* against their associated fungal, bacterial, and viral pathogens (van den Burg *et al.*, 2008).

Despite the large number of RING domain E3 ligases identified in plants, few have been implicated in defence signalling to date. The RING domain E3 Ub ligase ACRE132 was identified in the ACRE screen reported by Durrant *et al.* (2000). *ACRE132* is the proposed tobacco orthologue of the *Arabidopsis ATL2* gene, which is transcriptionally induced by fungal chitins during basal resistance, suggesting a possible conservation in function for these proteins in plant fungal response pathways (Delauré *et al.*, 2008).

Several studies have indicated a prominent role for U-box E3 Ub ligases during plant defence during both PTI and

ETI. Trujillo *et al.* (2008) recently reported the cumulative involvement of *Arabidopsis* U-box proteins PUB22, PUB23, and PUB24 (PLANT U-BOX 22–24) as negative regulators of basal resistance. In this study, single, double, and triple *pub22 pub23 pub24* mutants exhibited progressive loss of suppression in the flg22-induced ROI burst, MPK3 MAPK kinase activation, and downstream PTI marker gene expression (Trujillo *et al.*, 2008).

U-box E3 Ub ligases were also identified in the previously discussed ACRE screen, resulting in the implication of ACRE276/PUB17 and ACRE74/CMPG1 as positive regulators of ETI (Gonzalez-Lamothe *et al.*, 2006; Yang *et al.*, 2006). Gene silencing approaches demonstrated that tobacco ACRE276 is required for efficient HR development mediated by the *R* genes *Cf-9* and *N* and that the tomato ACRE276 orthologue is required for full resistance against *C. fulvum* (Yang *et al.*, 2006). PUB17, the *Arabidopsis* orthologue of ACRE276, was also implicated in defence, with *pub17* mutants demonstrating increased susceptibility against avirulent strains of *P. syringae* (Yang *et al.*, 2006). Similar experimental approaches have also demonstrated that the tobacco U-box protein CMPG1 mediates Cf-9-triggered HR and resistance (Gonzalez-Lamothe *et al.*, 2006). Mutant screening programmes in rice led to the identification of the lesion mimic mutant *spl11* which negatively regulates basal resistance against the rice pathogens *Magnoporthe grisea* and *Xanthomonas oryzae* (Yin *et al.*, 2000). Subsequent studies led to the characterization of SPL11 and demonstration of its *in vitro* activity as a functional U-box E3 Ub ligase (Zeng *et al.*, 2004).

RAR1/SGT1-mediated *R* gene resistance

The finding that several defence-associated E3 Ub ligases regulate disease resistance against distinct pathogen species supports the idea that multiple pathogen perception systems converge on common ubiquitination-based signalling pathways (Devoto *et al.*, 2003). The identification of RAR1 and SGT1 has defined one such convergence point between ubiquitination and resistance mediated by multiple *R* genes in monocot and dicot plant species (Muskett and Parker, 2003). *RAR1* encodes a predicted cytosolic protein of unknown function which contains two similar cysteine- and histidine-rich (CHORD) Zn^{2+}-binding domains (Shirasu *et al.*, 1999). RAR1 is conserved in all eukaryotes except yeast and was initially implicated in disease resistance against powdery mildew in barley mediated by the *R* genes *Mla6* and *Mla12* (Shirasu *et al.*, 1999). Plant RAR1 proteins were found to interact through their C-terminal CS motif with SGT1 (SUPRESSOR OF THE G2 ALLELE OF SKP1) which has multiple functions in yeast by association with several distinct protein complexes (Schadick *et al.*, 2002).

One function of SGT1 in yeast is to regulate SCF Ub E3 ligase complexes with which it associates through the SKP1 subunit (Kitagawa *et al.*, 1999). Similar interactions have been reported in *Arabidopsis*, barley, and *Nicotiana benthamiana* (Azevedo *et al.*, 2002). Association of SGT1 with the SCF complex in plants is supported by the finding that F-box-mediated auxin- and JA-dependent signalling is disrupted in *Arabidopsis sgt1b* mutants, suggesting that SGT1b is a key component of multiple SCF-regulated pathways (Gray *et al.*, 2003). Mutant analyses in *Arabidopsis* and silencing experiments in barley and *N. benthamiana* have demonstrated that SGT1 is required for responses that are mediated by diverse *R* gene structural types to induce resistance against a variety of pathogens (Azevedo *et al.*, 2002; Liu *et al.*, 2002; Peart *et al.*, 2002).

Additional evidence which supports the role of Ub-mediated degradation in defence signalling has come from silencing genes encoding SKP1 and subunits of the COP9 signalosome (CSN) in *N. benthamiana*, resulting in the loss of N-mediated tobacco mosaic virus (TMV) resistance (Liu *et al.*, 2002). The CSN is an evolutionarily conserved multiprotein complex which is closely related to the Lid subcomplex of the 26S proteasome that interacts with RAR1 and SGT1 and regulates ubiquitination by SCF E3 Ub ligases (Muskett and Parker, 2003).

SGT1 has also been shown to interact with HSP90 (HEAT SHOCK PROTEIN 90) which has been implicated in resistance mediated by several *R* genes (Takahashi *et al.*, 2003). Current research suggests that SGT1 and RAR1 associate as co-chaperones with HSP90 and are proposed to function in close proximity to R proteins, possibly to assist in the maintenance of conformation-sensitive signalling states during R protein activation (Shirasu and Schulze-Lefert, 2003). Collectively, SGT1 and RAR1 are thought to function in disease resistance through participation in multiple protein complexes where they are proposed to influence the conformation of *R* gene complexes and regulate ubiquitination by several classes of E3 ligase (Shirasu and Schulze-Lefert, 2003).

Target substrates of ubiquitination linked to plant defence

Beyond the reported interactions of RAR1 and SGT1 discussed above, relatively few defence-associated ubiquitination targets have been identified in plants. A potential link between ubiquitination and defence has been established in the case of the *Arabidopsis* R protein RPM1 which is degraded coincident with the onset of the HR elicited by *P. syringae* carrying the *avrRpm1*, *avrB*, *avrRps4*, or *avrRpt2* avirulence genes (Boyes *et al.*, 1998). RPM1 has been found to interact with the proteins RIN2 and RIN3 (RPM1-interacting proteins) which both demonstrate *in vitro* E3 Ub ligase activity and collectively contribute to pathogen-elicited RPM1-dependent ion leakage (Kawasaki *et al.*, 2005). HR-associated degradation of RPM1 is not altered in *rin2 rin3* double mutants, suggesting that whilst RIN2 and RIN3 are linked to defence signalling, they may not directly control RPM1 stability (Kawasaki *et al.*, 2005).

Manipulation of host ubiquitination signalling by several viral and bacterial plant pathogens which mimic host proteins to suppress defence and promote their own survival

have been reported (Dreher and Callis, 2007). The *P. syringae* effector protein avrPtoB represents one such example which functions to suppress immunity by inhibiting the plant HR. The Pst effectors avrPto and avrPtoB are delivered into the plant cell through the type III secretion system and are both recognized by the tomato resistance protein Pto to initiate HR and resistance (Pedley and Martin, 2003). In *N. benthamiana*, avrPtoB has been shown to be a suppressor of HR induced by avrPto/Pto recognition as well as HR induced by fungal elicitors and other bacterial effectors (Abramovitch *et al.*, 2003). AvrPtoB is a modular protein for which deletion and structural analysis has established that the C-terminal domain triggers HR whilst the N-terminal domain controls hypersensitive cell death suppression and possesses the structural features of a U-box E3 Ub ligase (Janjusevic *et al.*, 2006). The avrPtoB C-terminal domain exhibits *in vitro* E3 Ub ligase activity, and structural or catalytic mutations within this domain result in reduced HR suppression and virulence of *P. syringae in vivo* (Janjusevic *et al.*, 2006).

AvrPtoB uses its ligase activity to ubiquitinate and degrade the host R protein Fen, a Ser/Thr kinase that is able to interact physically with the N-terminal region of AvrPtoB that is lacking its C-terminal domain (Fig. 1) (Rosebrock *et al.*, 2007). The proposed relationship between avrPtoB, Fen, and Pto illustrates the evolving relationship between plant effectors and R proteins and how host ubiquitination can be exploited to benefit pathogen virulence (Rosebrock *et al.*, 2007). First, the pathogen avrPtoB (N-terminal domain only) evolved to suppress plant basal defences. Next, the plant Fen kinase evolved to bind avrPtoB (N-terminal domain only), leading to activation of *R* gene-mediated resistance. Subsequently, the pathogen responded by incorporating an E3 ubiquitin ligase domain into avrPtoB (forming full-length avrPtoB) that targets the Fen kinase for degradation. Finally, the plant kinase Pto, which is not susceptible to avrPtoB-mediated ubiquitination, evolved to bind avrPtoB, thus restoring host immunity through *R* gene-mediated resistance (Rosebrock *et al.*, 2007). Recently if was shown that avrPtoB can also affect PTI by ubiquitinating the PAMP receptor FLS2 for proteasomal degradation (Fig. 1) (Göhre *et al.*, 2008).

Opportunistic acquisition of host genetic material by pathogens such as *Agrobacterium tumefaciens* represents an alternative virulence strategy to the U-box structural mimicry demonstrated by avrPtoB which was generated through convergent evolution. *Agrobacterium tumefaciens* uses a type IV secretion system to translocate effectors and single-stranded DNA (T-DNA) into eukaryotic cells, resulting in genetic colonization of the host (Tzfira *et al.*, 2004). During infection, *A. tumefaciens* translocates the F-box protein VirF into host cells and utilizes host components to form a functional SCF complex required for degradation of VirE2 and host VIP1 (Schrammeijer *et al.*, 2001). VirE2 and VIP1 proteins must be eliminated to allow integration of the *Agrobacterium* T-DNA into the host genome (Fig. 1) (Tzfira *et al.*, 2004). VirF was the first prokaryotic protein reported to contain a conserved F-box domain (Schrammeijer *et al.*, 2001), and demonstrates the utilization of host functional domains obtained by lateral gene transfer to improve pathogen virulence.

Concluding remarks and future perspectives

As a system of protein degradation, the ubiquitin–proteasome system (UPS), which is widely and highly conserved, would be a highly suitable and adaptable mechanism to target foreign molecules for destruction; an effector molecule could quite simply be recognized and degraded before having time to act. Furthermore, due to the many tandem expansions in plants, this would serve to create more divergent *R* gene alleles. However, as the battle continues between the host and the pathogen, constant evolution has pushed the boundaries of ubiquitination beyond the confinement of the UPS.

Future directions of this work should focus on finding downstream targets and their ubiquitinated states during the defence response. For example, virtually nothing is known about non-K48-linked polyubiquitination in plant defence; could pathogen effector molecules acting as E3 ligases alter the fate of host defence molecules by switching Ub chain linkage? More in-depth knowledge about non-proteasome-associated ubiquitination will need to be acquired, with insight being provided by studies in other organisms such as yeast.

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