Ubiquitination during Plant Immune Signaling[1]

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Plant responses to pathogens depend on the rapid and effective coordination of microbial perception and downstream signal transduction events. Detection of pathogen invasion starts by the recognition of conserved microbial molecules called pathogen-associated molecular patterns (PAMPs), mainly by plant membrane-associated extracellular receptors, which results in PAMP-triggered immunity (PTI). Using a type III secretion system, plant pathogenic bacteria are able to inject type III effectors (T3Es) directly inside host cells, thereby overcoming PTI and favoring bacterial growth. Recognition of T3Es by plant resistance (R) proteins leads to effector-triggered immunity (ETI), a more efficient form of resistance that is regularly associated with the development of hypersensitive cell death (HR) at the site of pathogen penetration (Jones and Dangl, 2006). In addition, the onset of the HR typically triggers systemic acquired resistance (SAR), an inducible form of plant defense that spreads resistance to systemic tissues through mobilization of salicylic acid (SA)-mediated defenses and confers broad-spectrum immunity to secondary infection (Spoel and Dong, 2012). Plant hormones are crucial systemic signals that strongly influence the level of plant resistance. Indeed, significant changes in hormone levels and hormonal cross talk occur in plant cells interacting with microbes and are essential to the efficient integration of biotic stress cues (Pieterse et al., 2009).

The intricate molecular mechanisms that govern plant immune responses engage a high degree of proteomic plasticity to which posttranslational protein modification through ubiquitination contributes crucially. Ubiquitin is a small (8.5 kD) and highly conserved protein modifier that, covalently linked to target proteins, leads to their proteasomal degradation or to other fates including relocalization or endocytosis. Typically, proteins modified by sequential linkage of multiple ubiquitin residues via the ubiquitin residue Lys-48 are targeted for degradation by the 26S proteasome, a highly conserved proteolytic complex composed of two subparticles (Smalle and Vierstra, 2004): (1) the barrel-shaped 20S proteasome that is a stack of two outer rings formed by seven α-subunits ($\alpha1$–$\alpha7$) and two inner rings of seven β-subunits ($\beta1$–$\beta7$) enclosing a cavity with the active sites for protein degradation and (2) the 19S regulatory particles that are attached at both ends of the 20S cylinder and recognize the protein targeted for degradation.

The ubiquitin-26S proteasome system (UPS) involves the sequential action of three enzymes, namely E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligase), to ultimately ligate one or more ubiquitin molecules to specific target proteins (Vierstra, 2009). Ubiquitin is first activated for transfer by the E1 enzyme and activated ubiquitin is then transferred to a Cys residue in the E2. The ubiquitin-E2 intermediate generally serves as the proximal ubiquitin donor, using the E3 to identify the target and catalyze ubiquitin transfer. E3 enzymes are key factors that determine substrate specificity and are classified into four main subfamilies depending on their subunit composition and mechanism of action: Homologous to E6-associated protein Carboxyl Terminus (HECT), Really Interesting New Gene (RING), U-box, and cullin-RING ligases (CRLs; Vierstra, 2009). HECT proteins are single polypeptides that, unlike other E3 ligases, form a thioester intermediate with ubiquitin before ubiquitination of the target (Downes et al., 2003). RING and U-box proteins are structurally related single polypeptides that respectively use zinc chelation and hydrogen bonds/salt bridges to transfer ubiquitin from the E2-ubiquitin intermediate to the substrate (Stone et al., 2005; Yee and Goring, 2009). CRLs are multisubunit E3 ligases that contain a cullin, a RING-BOX1 that binds to ubiquitin, and a variable module for target recognition (Vierstra, 2009). The modular S-phase Kinase-associated Protein1 (SKP1)/CULLIN1 (CUL1)/F-Box (SCF) group is the best-characterized CRL. Arabidopsis (*Arabidopsis thaliana*) SKP1-like proteins are known as Arabidopsis SKP1-like (ASK). In SCF complexes, CUL1 acts as a molecular scaffold by interacting at its C terminus with RING-BOX1 (which is linked to the E2-ubiquitin intermediate) and at the N terminus with SKP1/ASK (which is linked to the F-box protein, responsible for recruiting the target) thereby

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promoting the transfer of ubiquitin from the E2 to the target (Vierstra, 2009).

Here, we discuss the involvement of different UPS-related components during the regulation of plant immune responses, paying special attention to the well-characterized family of E3 ubiquitin ligases. Strikingly, targeted protein turnover through the UPS is a shared feature by most hormone signaling pathways (Santner and Estelle, 2009; Vierstra, 2009). Due to space limitations, we refer the reader to recently published reviews for a detailed account on the control of hormone signaling by ubiquitination during plant immunity (Trujillo and Shirasu, 2010; Robert-Seilaniantz et al., 2011). Finally, we also review our current knowledge about the exploitation of the host UPS by plant parasite proteins.

UBIQUITIN AND 26S PROTEASOMAL COMPONENTS

Direct studies involving ubiquitin gene disruption may lead to severe effects in plant performance and eventually to plant lethality. To counteract this problem, several studies have used ubiquitin variants or transient silencing strategies. The use of a ubiquitin variant containing Arg instead of Lys at position 48 (UbR48) impairs polyubiquitination, and thus proteolytic degradation, but allows monoubiquitination to occur. Expression of UbR48 in tobacco (*Nicotiana tabacum*) plants induced the development of necrotic lesions and altered plant responses to infection with *Tobacco mosaic virus* (TMV; Becker et al., 1993), whereas in Arabidopsis, expression of UbR48 led to spontaneous cell death symptoms, reactive oxygen species (ROS) production, and constitutive induction of defense-related genes (Schlögelhofer et al., 2006). However, Arabidopsis plants expressing UbR48 did not present altered resistance to virulent or avirulent *Pseudomonas syringae* bacterial strains (Schlögelhofer et al., 2006). Partial depletion of ubiquitin levels by transient-induced silencing of the ubiquitin-encoding gene in barley (*Hordeum vulgare*) epidermal cells resulted in enhanced susceptibility to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Dong et al., 2006). Moreover, complementation studies suggested a role for Lys-48-linked polyubiquitination in defense signaling (Dong et al., 2006).

The importance of proteasomal subunits for the regulation of the plant response to microbes has also been documented. In tobacco, expression of three genes encoding subunits of the 20S proteasome (α3, α6, β1) is induced after treatment with the elicitor cryptogein (Dahan et al., 2001; Suty et al., 2003). Tobacco cell lines overexpressing the β1 subunit showed a drastic reduction of the *NtRbohD* (NADPH oxidase) gene induction and of its associated oxidative burst after cryptogein treatment, suggesting that the β1 subunit acts as a negative regulator of early plant responses to cryptogein (Lequeu et al., 2005). In addition, RNA interference (RNAi) stable Arabidopsis lines against the proteasome β1 subunit displayed altered cell death responses against bacterial pathogens (Hatsugai et al., 2009). Proteasome subunit-regulated cell death was associated with the fusion of the central vacuole with the plasma membrane, discharging vacuolar antibacterial proteins to the outside of the cells where bacteria proliferate. Interestingly, this response was shown by plants infected with avirulent but not virulent bacteria, suggesting that this type of cell death is related to *R*-gene-mediated resistance (Hatsugai et al., 2009). In this context, proteasome subunit-mediated protein degradation appears to be required for cell death but not for defense signaling, as cell death was not accompanied by altered ROS production or expression of defense-related genes (Hatsugai et al., 2009).

In contrast to the manipulation of the 20S core subunits, systematic RNAi of 40 genes encoding all 17 subunits of the 19S proteasome regulatory subcomplex did not modify the defense response against *B. graminis* f. sp *hordei* (Dong et al., 2006). These data suggest that the role played by Lys-48-linked protein polyubiquitination in barley basal defense is independent from the proteasome pathway.

Altogether, the proteasome-dependent defense appears to be involved in defense responses against viruses and in *R*-gene-related resistance against bacterial pathogens, but not in basal host defense against fungal pathogens, thus suggesting that the proteasome may be involved in susceptibility rather than in basal defense.

THE UBIQUITIN CONJUGATION SYSTEM

E1 Activating Enzymes

Two E1 enzymes (UBA1, UBA2) have been described in Arabidopsis. The Arabidopsis *modifier of snc1-5* (*mos5*) mutant carries a 15-bp deletion in the *AtUBA1* gene, which suppresses *suppressor of npr1-1 constitutive1* (*snc1*)-mediated resistance (Goritschnig et al., 2007). *snc1* plants carry a point mutation in an *R* gene that results in constitutive activation of defense responses (Li et al., 2001; Zhang et al., 2003). *mos5* mutant plants were more susceptible to a virulent *Pseudomonas* strain and displayed differential susceptibility when inoculated with bacteria carrying different avirulence factors (Goritschnig et al., 2007). This suggests a role of functional ubiquitination machinery in basal defense against bacterial pathogens and a specific role of this E1 in some, but not all, R-protein-mediated resistance responses. Interestingly, although mutation of *UBA2* did not suppress *snc1*-mediated resistance, the double mutant *mos5uba2* was lethal, suggesting partial redundancy of the two E1 enzymes and a differential requirement for Arabidopsis disease resistance (Goritschnig et al., 2007). In tobacco, expression of *NtUBA1* and *NtUBA2* was induced in response to viral infection, wounding, and defense-related hormones

such as SA and jasmonic acid and the ethylene precursor 1-aminocyclopropane-1-carboxylic-acid (Takizawa et al., 2005).

E2 Conjugating Enzymes

Based on the induction of their expression following elicitation, E2 conjugating enzymes have been suggested to contribute to plant disease resistance. For example, expression of the rice (*Oryza sativa*) E2-encoding gene *OsUBC5b*, but not its homolog *OsUBC5a*, was induced in suspension-cultured rice cells treated with *N*-acetylchitooligosaccharide elicitor (Takai et al., 2002). Both enzymes are able to catalyze autoubiquitination of EL5, a RING-type E3 ligase that is also induced upon elicitor treatment (see below; Takai et al., 2001, 2002). However, direct involvement of E2 proteins in plant defense responses remains to be demonstrated.

E3 Ligases

RING Proteins

Four-hundred and seventy-seven genes encode RING-type proteins in Arabidopsis. Modulation of the expression of *RING* genes following different elicitation treatments has been described (Durrant et al., 2000; Navarro et al., 2004; Ramonell et al., 2005; Zipfel et al., 2006). However, the involvement in immunity of RING proteins has only been demonstrated for a few of them and, in most cases, their substrates remain to be identified.

Members of the *ARABIDOPSIS TóXICOS EN LEVADURA* (*ATL*) gene family of RING zinc-finger E3 ubiquitin ligases are activated by elicitor treatment and play important roles in defense pathways. Expression of the tobacco *ATL* gene *AVR9/CF-9-RAPIDLY ELICITED132* (*ACRE132*) was induced during the defense response triggered following specific recognition of the fungal Avr9 effector by the resistance protein Cf-9 (Durrant et al., 2000). Expression of Arabidopsis *ATL2*, *ATL6*, *ATL9*, *LeATL6* (for the tomato [*Solanum lycopersicum*] ortholog of Arabidopsis ATL6), and rice *EL5* was rapidly induced in response to elicitor treatment (Salinas-Mondragón et al., 1999; Takai et al., 2002; Serrano and Guzmán, 2004; Ramonell et al., 2005; Hondo et al., 2007; Berrocal-Lobo et al., 2010). In addition, constitutive expression of *ATL2* in Arabidopsis led to induced defense-related gene expression (Serrano and Guzmán, 2004). Finally, an *atl9* loss-of-function mutant displayed increased susceptibility to *Golovinomyces cichoracearum* (Ramonell et al., 2005). ATL9 is an active E3 ubiquitin ligase localized to the endoplasmic reticulum. Interestingly, ATL9 expression appeared to be dependent on NADPH oxidases and mutation in *ATL9* compromised the production of ROS

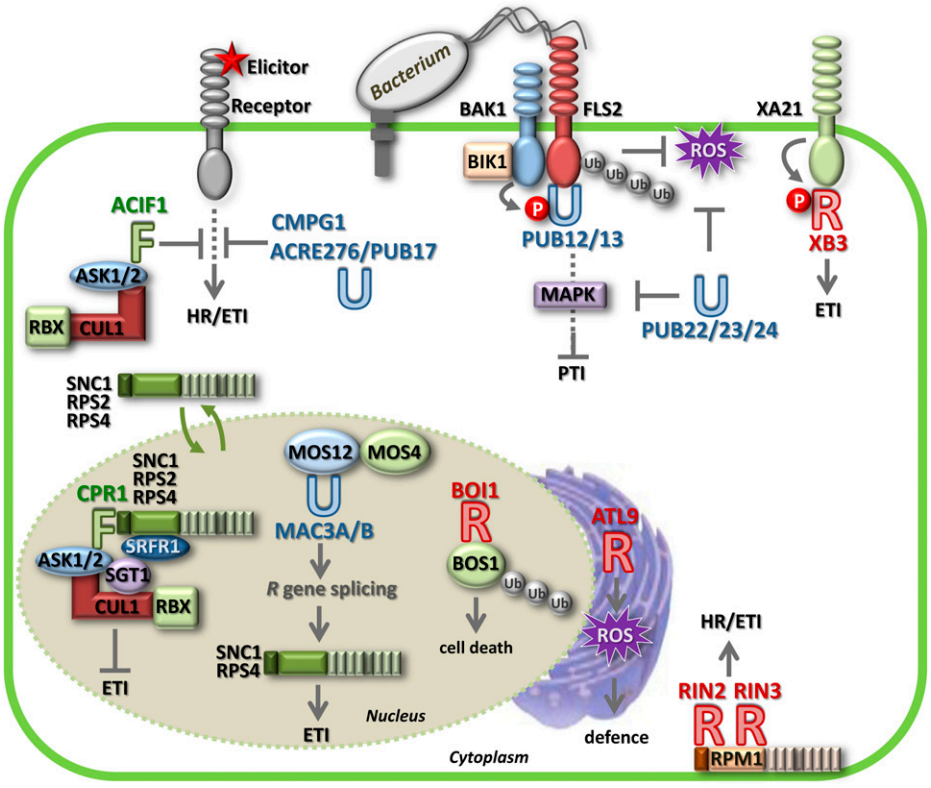

Figure 1. Schematic representation of some of the best-characterized plant E3 ubiquitin ligase proteins involved in the regulation of plant immune responses. RING, U-box, and F-box proteins are respectively represented by red R, blue U, and green F symbols. SNC1 and RPS4 R proteins are localized both in the cytoplasm and the nucleus (green arrows represent R-protein nucleocytoplasmic shuttling). Although the nuclear localization of RPS2 has not been documented, this R protein is represented together with SNC1 and RPS4 because of the similarities in terms of the regulation of the stability of the three R proteins. It is possible that the represented protein complexes also occur in the cytoplasm. See the text for details.

after infection (Berrocal-Lobo et al., 2010). These data, together with expression profiling analysis of the *atl9* mutant after chitin treatment, revealed a complex interplay between chitin-mediated oxidative burst and defense pathways (Berrocal-Lobo et al., 2010; Fig. 1).

In Arabidopsis, the R2R3-type MYB transcription factor BOTRYTIS SUSCEPTIBLE1 (BOS1) is required for resistance to pathogens and for tolerance to certain abiotic stresses (Mengiste et al., 2003). The RING E3 ligase protein BOS1 INTERACTOR1 (BOI1) physically interacted with BOS1 in the plant cell nucleus and was able to ubiquitinate BOS1 in vitro (Luo et al., 2010; Fig. 1). Similar to the *bos1* mutant (Mengiste et al., 2003), *BOI1* RNAi Arabidopsis plants were more susceptible to infection by *Botrytis cinerea* and less tolerant to salt stress compared with wild-type plants. In addition, *BOI1* RNAi plants overexpressing BOS1 displayed enhanced resistance to *B. cinerea* and tolerance to oxidative stress, suggesting that BOS1 may be a target of BOI1. However, considering the phenotype of *BOI1* RNAi Arabidopsis plants, in which accumulation of the BOS1 protein should be expected, the effect of BOI1 on BOS1 protein accumulation remains unclear. BOS1 protein expression in planta was only detectable following treatment with the proteasome inhibitor MG132, both in wild-type and *BOI1* RNAi plants, suggesting that the protein is rapidly turned over (Luo et al., 2010). The authors suggested that residual BOI1 protein in *BOI1* RNAi plants may be sufficient to lead to BOS1 degradation or that functional redundancy may exist with additional closely related E3 ligase proteins. In addition, it is also possible that during salt/oxidative stress and *B. cinerea* infection, BOS1 may be stabilized to confer tolerance to stress, consistent with previous reports describing stress-induced protein stabilization to promote stress tolerance (Luo et al., 2010). Finally, BOI1 was shown to be required for the regulation of some, but not all, types of cell death. For example, BOI1 restricted the extent of cell death induced by both the fungal toxin α-picolinic and virulent *Pseudomonas* but did not affect ETI-related HR responses triggered by avirulent *Pseudomonas* strains (Luo et al., 2010).

The R2R3-type MYB transcription factor AtMYB30 is a positive regulator of plant defense and HR responses (Raffaele et al., 2008). Negative regulation of AtMYB30-mediated defense through its interaction with the secreted phospholipase *At*sPLA$_2$-α was previously reported (Froidure et al., 2010). A second mechanism for negative regulation of AtMYB30 activity was uncovered by the finding that AtMYB30 interacts with a RING E3 ligase protein that is able to ubiquitinate AtMYB30, leading to its degradation by the proteasome (S. Rivas, unpublished data).

The Arabidopsis RING proteins RIN2 and RIN3 interact with the R protein RPM1, a peripheral plasma membrane protein that confers resistance to *P. syringae* expressing AvrRpm1. RPM1 disappears at the onset of the HR through an unknown proteasome-dependent mechanism (Boyes et al., 1998; Kawasaki et al., 2005). As RPM1, RIN2, and RIN3 were predominantly localized to the plasma membrane (Fig. 1). Inoculation with *P. syringae* expressing AvrRpm1 or AvrRpt2 induced (1) reduced RIN2 electrophoretic mobility before the appearance of the HR and (2) disappearance of a major part of RIN2 at the time of the HR. A *rin2rin3* double mutant displayed reduced RPM1- and RPS2-dependent HR, although RPM1 disappearance and pathogen growth were not modified in these plants (Kawasaki et al., 2005). Indeed, although RIN2 and RIN3 are active E3 ubiquitin ligases, they failed to ubiquitinate RPM1 in vitro, suggesting that they act on a substrate that regulates RPM1- and RPS2-dependent HR (Kawasaki et al., 2005).

Expression of Arabidopsis *RING1* was up-regulated by treatment with the fungal toxin fumonisin B1 as well as after inoculation with an avirulent *Pseudomonas* strain (Lin et al., 2008). RING1 was associated to lipid rafts of plasma membranes and was shown to display E3 ligase activity in vitro (Lin et al., 2008). Silencing of RING1 using an artificial microRNA resulted in fumonisin B1 hyposensitivity and reduced expression of the defense marker gene *PATHOGENESIS-RELATED1* (*PR1*). Together, these data suggest that RING1 may be involved in the regulation of plant defenses perhaps though degradation of a plasma membrane-associated negative regulator of cell death (Lin et al., 2008).

The *benzoic acid hypersensitive1-Dominant* (*bah1-D*) Arabidopsis mutant carries a mutation in a RING-type ubiquitin E3 ligase protein (BAH1) and accumulated excess amounts of the plant hormone SA after treatment with the SA precursor benzoic acid and after inoculation with virulent *Pseudomonas* (Yaeno and Iba, 2008). *bah1-D* is allelic to the *nla* mutant, which has been shown to exhibit early senescence under low-nitrogen conditions (Peng et al., 2007). *bah1-D* exhibited localized cell death after infection with virulent bacteria and this phenotype was dependent on SA accumulation, whereas age-related cell death appeared to be independent of SA (Yaeno and Iba, 2008).

In rice, several RING-type E3 ligase proteins have been involved in plant defense responses against pathogen infection. Expression of the rice RING zinc-finger protein OsRHC1 in Arabidopsis conferred improved resistance to virulent bacteria and this phenotype was inhibited by the proteasome inhibitor MG132 (Cheung et al., 2007). In addition, expression of rice *BLAST AND BTH-INDUCED1* (*OsBBI1*) was induced by the rice blast fungus *Magnaporthe oryzae*, as well as by the chemical inducers benzothiadiazole and SA (Li et al., 2011). OsBBI1 is an active RING-type E3 ligase that was found to mediate broad-spectrum disease resistance against the blast fungus by modifying cell wall defense responses. Indeed, OsBBI1-overexpressing plants accumulated hydrogen peroxide and phenolic compounds and displayed enhanced cross-linking of proteins in cell walls at infection sites by *M. oryzae* compared with wild-type plants (Li et al., 2011). The rice receptor-like kinase protein XA21 confers

resistance to *Xanthomonas oryzae* pv *oryzae* (*Xoo*), the causal agent of bacterial blight disease. XA21-BINDING PROTEIN3 (XB3) is an active RING-type E3 ubiquitin ligase that binds to the kinase domain of XA21 through an ankyrin repeat domain and is substrate for the XA21 kinase activity in vitro (Wang et al., 2006; Fig. 1). Transgenic plants with reduced *XB3* expression presented decreased levels of the XA21 protein and were compromised in resistance to the avirulent race of *Xoo*, indicating that XB3 is necessary for full accumulation of XA21 and for XA21-mediated resistance (Wang et al., 2006). Interestingly, interaction of E3 ligase proteins with the kinase domain of RLKs appears to be a conserved feature for the regulation of various plant processes (Kim et al., 2003; Samuel et al., 2008; Lu et al., 2011).

In pepper (*Capsicum annuum*), expression of two genes encoding RING-type proteins, *CaRING1* and *CaRFP1*, was induced by an avirulent strain of *Xanthomonas campestris* pv *vesicatoria* (Hong et al., 2007; Lee et al., 2011). CaRING is an active E3 ligase that localizes to the plasma membrane and is required for HR and resistance responses in pepper to infection with virulent and avirulent strains of *X. campestris* pv *vesicatoria*. In addition, CaRING1 overexpression in Arabidopsis induces enhanced resistance to *Pseudomonas* and *Hyaloperonospora arabidopsidis* (Lee et al., 2011). CaRFP1 physically interacts with the basic PR1 protein CABPR1 (Hong et al., 2007). *CaRFP1* expression was additionally induced in pepper leaf tissues infected by the fungus *Colletotrichum coccodes* and following treatment by several defense-related hormones and abiotic stresses (Hong et al., 2007). CaRFP1 overexpression in Arabidopsis conferred disease susceptibility to *Pseudomonas* infection, accompanied by suppression of SA-dependent signaling and altered responses to osmotic stress and abscisic acid (Hong et al., 2007). These results suggest that CaRFP1 may act as an early defense regulator controlling bacterial disease susceptibility and tolerance to osmotic stress, although whether CaRFP1 is an active E3 ligase and CABPR1 is a target of this activity remains to be demonstrated.

U-Box Proteins

The rice lesion mimic mutant *spotted leaf11* (*spl11*) displayed a spontaneous cell death phenotype and enhanced resistance to *Magnaporte grisea* and *Xoo* (Yin et al., 2000). *SPL11* encodes a U-box/ARMADILLO repeat protein (Zeng et al., 2004). Expression of *SPL11* was induced by both incompatible and compatible rice-blast interactions (Zeng et al., 2004). Although SPL11 has been shown to display E3 ubiquitin ligase activity (Zeng et al., 2004), the molecular mechanism by which SPL11 is able to modulate plant defense signaling remains unknown. The Arabidopsis ortholog of SPL11, PUB13 (for Plant U-box), was recently shown to regulate cell death, defense responses, and flowering time (Li et al., 2012). It has been therefore suggested that SPL11/PUB13 represents a convergence point of defense and flowering signaling in plants (Liu et al., 2012). Similarly to rice *spl11*, *pub13* mutant plants displayed spontaneous cell death and this phenotype was complemented by overexpression of SPL11. *pub13* plants presented increased resistance against biotrophic bacterial, fungal, and oomycete pathogens. In contrast, *pub13* plants were susceptible against infection by necrotrophic fungi (Li et al., 2012). PUB13-mediated defense responses were dependent on SA signaling. According to the previous observation that high humidity enhances lesion mimic phenotypes for some mutants (Lorrain et al., 2003), *pub13* cell death and resistance phenotypes, as well as susceptibility to necrotrophic pathogens, were enhanced under high humidity (Li et al., 2012).

Flagellin perception by the flagellin receptor FLAGELLIN SENSING2 (FLS2) leads to FLS2 association with the coreceptor protein BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1; Chinchilla et al., 2007). Following flagellin treatment, the U-box proteins PUB12 and PUB13 were found to be recruited to the FLS2 receptor complex in a BAK1-dependent manner (Lu et al., 2011). BAK1 phosphorylated both PUB12 and PUB13 and this phosphorylation was enhanced in presence of flagellin. BIK1, an FLS2/BAK1 associated kinase, was not able to phosphorylate PUB12/13 but stimulated the capacity of BAK1 to phosphorylate PUB13 (Lu et al., 2011). PUB12 and 13 are active E3 ligases able to ubiquitinate in vitro FLS2 but not BAK1 (Fig. 1). FLS2 ubiquitination by PUB12/13 is consistent with the previous finding that flagellin promotes the translocation of FLS2 to vesicles, which is followed by FLS2 degradation (Robatzek et al., 2006). However, PUB12/13 were able to ubiquitinate FLS2 deleted from its PEST domain, which is necessary for its internalization, thus suggesting that ubiquitination and internalization are uncoupled (Lu et al., 2011). PUB12 and PUB13 were not required for flagellin perception but plant responses to flagellin were enhanced in *pub12* or *pub13* mutant plants. Resistance to bacterial infection, although not modified in *pub12* or *pub13* single mutants under the conditions tested, was enhanced in the double mutant *pub12pub13*. This was consistent with the absence of FLS2 degradation in *pub12pub13* plants after flagellin treatment, suggesting functional redundancy between both proteins. Altogether, this work illustrates a negative regulatory mechanism of flagellin-related defense responses via the ubiquitination-mediated turnover of FLS2, which depends on BAK1-mediated recruitment and phosphorylation of two E3 ligase proteins (Lu et al., 2011).

U-box proteins PUB22, 23, and 24 represent an additional group of negative regulators of PTI responses in Arabidopsis (Trujillo et al., 2008). Expression of these three genes was highly induced upon flagellin treatment and after inoculation with virulent *Pseudomonas* or *H. arabidopsidis*. As previously shown for PUB12 and 13, PUB22, 23, and 24 displayed a certain degree of functional redundancy since single, double,

and triple mutants exhibited progressive loss of suppression of flagellin-induced defense signaling. In addition, triple-mutant plants displayed enhanced resistance to inoculation with *P. syringae* and *H. arabidopsidis* (Trujillo et al., 2008). Interestingly, plant responses to different PAMPs were also enhanced in the triple mutant, suggesting a shared mechanism of down-regulation of PTI signaling in response to distinct PAMPs through PUB22, 23, and 24 (Trujillo et al., 2008; Fig. 1).

Two genes encoding U-box proteins, *ACRE74/CMPG1* and *ACRE276*, were rapidly induced in tobacco cell cultures expressing the tomato resistance gene *Cf-9* after elicitation with its cognate avirulence protein from the fungal pathogen *Cladosporium fulvum* (Durrant et al., 2000). In contrast to SPL11/PUB13, NtCMPG1 and NtACRE276 are positive regulators of the HR and resistance in response to pathogen infection, in both tobacco and tomato (González-Lamothe et al., 2006; Yang et al., 2006). Expression of the Arabidopsis *CMPG1* homologs *PUB21* and *PUB22* was also induced after elicitor treatment or pathogen infection (Navarro et al., 2004). In general, CMPG1 activity was required for cell death triggered by perception of elicitors at the plasma membrane but appeared to be dispensable for cell death following recognition of cytoplasmic effectors by NBS-LRR proteins (González-Lamothe et al., 2006; Gilroy et al., 2011; Fig. 1). The Arabidopsis ACRE276 homolog PUB17 rescued the HR in *ACRE276*-silenced tobacco plants and Arabidopsis *pub17* mutant plants demonstrated increased susceptibility against avirulent but not virulent *Pseudomonas* strains (Yang et al., 2006). Together, these data demonstrate that both ACRE276/PUB17 and CMPG1 are positive regulators of ETI responses, which are required for full plant resistance to avirulent pathogens.

An additional example of U-box proteins acting as positive regulators of defense responses is provided by MAC3A and MAC3B, two U-box E3 ligases that are required for full basal and R-protein-mediated resistance in Arabidopsis (Monaghan et al., 2009). MAC3A and MAC3B are members of the MOS4-Associated Complex (MAC) and present high homology to the yeast (*Saccharomyces cerevisiae*) and human Prp19 ubiquitin ligases, involved in RNA processing (Palma et al., 2007). Like other *mos* mutants, *mos4* alleles were able to suppress *snc1*-mediated resistance. Both MAC3A and MAC3B were required to raise an effective defense response. Indeed, *mac3a* and *mac3b* single mutants were not compromised in basal defense responses while the double mutant exhibited enhanced susceptibility to virulent bacteria and to some but not all avirulent strains tested. Thus, MAC3A and MAC3B play redundant roles and are required for signaling pathways mediated by specific R proteins (Monaghan et al., 2009). A recent report showed that mutation of an additional *MOS* gene, *MOS12*, encoding an Arg-rich protein homologous to human cyclin L, resulted in altered splicing patterns of *SNC1* and *RPS4* and reduced levels of these R proteins (Xu et al., 2012). MOS12 interacts with the MAC in the nucleus, indicating that MOS12 and the MAC are required for the fine tuning of *R* gene expression via the splicing machinery, in a process that appears to be critical for directing appropriate defense outputs (Fig. 1).

F-Box Proteins

F-box proteins confer substrate specificity within SCF complexes. Since the F-box superfamily is one of the largest and most diverse gene families in the plant kingdom, with approximately 700 members in Arabidopsis, a pervasive role of F-box proteins in the control of plant protein abundance has been proposed (Xu et al., 2009; Hua et al., 2011). In the context of plant defense responses, a prominent role of F-box proteins in the regulation of hormone signaling pathways has been extensively characterized and reviewed elsewhere (Trujillo and Shirasu, 2010; Robert-Seilaniantz et al., 2011).

Beyond their well-documented control of hormone production, F-box proteins have also been shown to play additional roles during the regulation of plant defense to pathogens. For example, expression of the rice F-box encoding gene *OsDRF1* was enhanced upon treatment with benzothiadiazole, a chemical inducer of defense responses, and the plant hormone abscisic acid (Cao et al., 2008). Overexpression of OsDRF1 in tobacco resulted in increased abscisic acid sensitivity and enhanced resistance against viral and bacterial inoculation.

SUPPRESSOR OF NIM1-1 (SON1) is another F-Box protein that has been involved in the regulation of Arabidopsis SAR, a form of defense that is regulated by SA and by the NIM1/NPR1 protein (Kim and Delaney, 2002). *nim1-1* mutants were highly susceptible to infection by the oomycete *H. arabidopsidis*. The *son1* mutant showed SAR-independent restoration of resistance against both *H. arabidopsidis* and *P. syringae*. Resistance in *son1* also was observed in a *NahG* background, in which SA is converted to catechol, indicating that it does not require accumulation of SA (Kim and Delaney, 2002).

Mutation of the Arabidopsis F-box encoding gene *CONSTITUTIVE PR1* (*CPR1/CPR30*) led to constitutive defense responses to *P. syringae* and dwarfism (Gou et al., 2009). The *cpr1* mutant presented a similar phenotype to the *bonzai1* (*bon1*) mutant, which carries a mutation in a copine gene, and this phenotype was strengthened in the double mutant *cpr1/bon1*, suggesting a synergistic interaction between both genes (Gou et al., 2012). Strikingly, this phenotype was rescued at 28°C, suggesting that *R* genes, which often display temperature-sensitive phenotypes, may mediate the *cpr1/bon1* phenotype. Indeed, consistent with the fact that expression of the *R* gene *SNC1* is upregulated in *bon1* mutant plants (Yang and Hua, 2004), a *snc1* mutation largely rescued the *cpr1* and *cpr1/bon1* phenotypes. Thus, SNC1 appeared to be a common

target of BON1 and CPR1, which respectively suppressed the accumulation of SNC1 transcripts and protein (Cheng et al., 2011; Gou et al., 2012). Indeed, CPR1 has been shown to control the accumulation of SNC1 and RPS2 in *Nicotiana benthamiana* in a proteasome-dependent manner (Gou et al., 2012). In Arabidopsis, loss-of-function mutations in *CPR1* led to higher accumulation of SNC1 and RPS2, as well as autoactivation of immune responses, which can be largely suppressed by mutation of *SNC1*, while overexpression of CPR1 rescued *bon1-1* and *snc1-1* mutant phenotypes (Cheng et al., 2011; Gou et al., 2012). Furthermore, CPR1 interacted with SNC1 and RPS2 in Arabidopsis protoplasts, and overexpressing CPR1 resulted in reduced accumulation of SNC1 and RPS2, as well as in suppression of immunity mediated by these two R proteins (Cheng et al., 2011). Therefore, the F-box protein CPR1 targeted SNC1 and RPS2 for degradation, thereby regulating their protein levels and preventing autoimmunity. SNC1 and RPS4 were also subject to negative regulation by SUPPRESSOR OF RPS4-RLD1 (SRFR1), a tetratricopeptide repeat protein with similarity to nuclear transcriptional repressors (Kwon et al., 2009; Kim et al., 2010; Li et al., 2010). Interestingly, SRFR1 interacted with SUPPRESSOR OF THE G2 ALLELE OF SKP1 (SGT1) whereas CPR1 interacted with multiple ASK proteins (Gou et al., 2009; Li et al., 2010). Since the SGT1 isoform SGT1b directly interacts with SKP1 and cullin proteins (Kitagawa et al., 1999; Azevedo et al., 2002), it is tempting to speculate that SRFR1 and SGT1b work together with the SCF^{CPR1} complex to regulate SNC1 and RPS4 protein stability (Fig. 1). The study of SNC1, RPS2, and RPS4 illustrates the tight and intricate control exerted on levels of immune receptors to prevent constitutive defense activation under nonpathogenic conditions.

Avr9/Cf-9-INDUCED F-Box1 (ACIF1; ACRE189) is an F-box protein with a Leu-rich-repeat domain found in a screen to identify proteins involved in Cf9-mediated ETI in *N. benthamiana* (Rowland et al., 2005). ACIF1 interacted with SCF subunits ASK1/2 and CUL1. In tobacco, silencing of ACIF1 compromised the HR triggered by various elicitors as well as the resistance response to TMV infection that is mediated by the *N* resistance gene and in cell death triggered by *P. syringae* (van den Burg et al., 2008; Fig. 1). Notably, expression of ACIF1 F-box catalytic mutants in tobacco compromised the HR, similarly to *ACIF1* silencing. In tomato, silencing of *ACIF1* attenuated the Cf-9-dependent HR and resistance to *C. fulvum* conferred by the Cf-9 homolog Cf-9B, although Cf-9-mediated resistance was not compromised. ACIF1 is widely conserved and is closely related to F-box proteins that regulate plant hormone signaling in Arabidopsis. Silencing of ACIF1 Arabidopsis homologs (VFBs) induced a subset of methyl jasmonate- and abscisic acid-responsive genes, supporting a regulatory role of ACIF1/VFBs in hormone-mediated plant defense responses (van den Burg et al., 2008).

EXPLOITATION OF THE HOST UPS BY MICROBIAL EFFECTORS

Bacterial E3 Ubiquitin Ligases

Bacterial effectors are the most-studied group of virulence determinants of any group of plant parasites. Bacteria are also known for not harboring any housekeeping UPS. Nevertheless, over the last years several bacterial T3E or type IV effectors have been identified directly functioning as ubiquitin ligases or promoting ubiquitin ligation. They either originate from ancient lateral transfer (cases of the F-box protein VirF or GALAs; Tzfira et al., 2004; Kajava et al., 2008) or have emerged though convergent evolution to give rise to sequence divergent but functionally conserved ubiquitin ligases (cases of AvrPtoB, and probably the IpaH homologs; Janjusevic et al., 2006; Singer et al., 2008). Functional homologs of plant F-box proteins have been characterized in *Agrobacterium tumefaciens* (VirF) and *Ralstonia solanacearum* (GALAs) and can be found on the genome of both *Xanthomonas* sp. and *P. syringae* (see PF00646 at http://pfam.sanger.ac.uk). Although not fully required for pathogenicity, VirF has been shown to interact with both the *A. tumefaciens* VirE2 and plant VIP1 proteins. VIP1 is destabilized by VirF in an SKP1-like dependent manner, suggesting that VirF participates in an SCF complex (Fig. 2). Although direct evidence is lacking, VIP1 instability further destabilizes VirE2, uncoating the T-DNA as it is imported into the nucleus (Tzfira et al., 2004). Since VIP1 binds both nucleosomes and the VirE2-coated T-DNA, it seems that VirF should have a central role in the gene transfer at the heart of *A. tumefaciens* parasitism. Consistent with the observation that VirF is not essential for infection of some plant species (Hirooka et al., 1987), a recent report showed that *Agrobacterium* infection induces the expression of VBF, a plant F-box protein capable of functionally replacing VirF and destabilizing VIP1 and VirE2 (Zaltsman et al., 2010). Interestingly *A. tumefaciens* uses another type IV effector, VirD5, to prevent VirF destabilization by the host UPS (Magori and Citovsky, 2011). Indeed, a plant SCF complex appears to be at least partly responsible for VirF degradation in host cells (Fig. 2; Magori and Citovsky, 2011). Another report confirmed that *Agrobacterium* transformation capability depends on the availability of the plant ASK1/2 SCF complex subunits and needs the SCF-associated proteins SGT1 and RAR1 (Anand et al., 2012). Furthermore, *A. tumefaciens* transformation is accompanied by induced expression of several plant F-box encoding genes. It was therefore speculated that these proteins may be involved in the protein destabilization processes mentioned earlier (Anand et al., 2012).

R. solanacearum strains contain between six and eight F-box proteins named GALAs that are differentially required for full pathogenicity in different host plants (Remigi et al., 2011). Although GALA ubiquitination

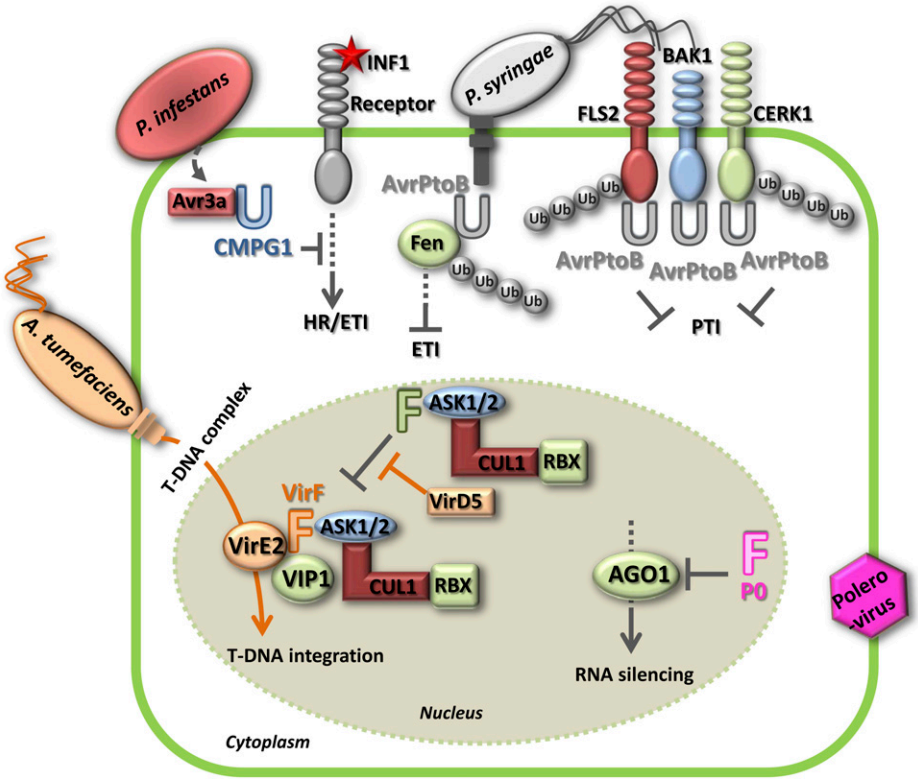

Figure 2. Schematic representation of some pathogen effectors interfering with the plant UPS. U-box and F-box effector proteins interfering directly or indirectly with the host UPS are color coded according to the pathogenic organism and respectively represented by U and F symbols. Plant U-box and F-box proteins are respectively represented by blue U and green F symbols. See the text for details.

targets remain to be identified, it is likely that their function in virulence is related to their putative E3 ubiquitin ligase activity. Indeed the F-box domain is essential for GALA7 virulence function on *Medicago truncatula* (Angot et al., 2006).

As parasite virulence effectors sometimes do not harbor any sequence homologies, solving their structure has proven seminal in understanding their function. For example, the structure of the *Shigella flexnerii* T3E IpaH was a first hint into its E3 ubiquitin ligase function. Interestingly both *P. syringae* and *R. solanacearum* contain IpaH homologs, which are related to HECT-type E3 ubiquitin ligases but their function(s) inside host cells remain to be determined (Singer et al., 2008).

Another important example of a probable convergent evolution event is AvrPtoB from *P. syringae*. This T3E presents structural homology to RING-finger and U-box E3 ubiquitin ligase proteins and displays a classical autocatalytic ubiquitin ligase activity (Janjusevic et al., 2006). Remarkably, the same bacterial protein harbors PTI and ETI suppression functions (Rosebrock et al., 2007) and, in an elegant coevolution scenario, illustrates all axes of the classical Zig-Zag scheme (Jones and Dangl, 2006). Indeed, AvrPtoB is capable of inhibiting PTI by multiple means. It targets the PRR receptors Chitin Elicitor Receptor Kinase1 and FLS2 for ubiquitination and degradation (Fig. 2; Göhre et al., 2008; Gimenez-Ibanez et al., 2009). Furthermore, apparently in an E3 ligase-independent fashion, AvrPtoB also targets the coreceptor protein BAK1 (Fig. 2; Shan et al., 2008) and interferes with MAPK signaling downstream of FLS2 activation (He et al., 2006). Finally, AvrPtoB also interferes with ETI since it recognizes and degrades the plant resistance protein Fen, a key player in the Pto/Prf-mediated resistance (Fig. 2; Oh and Reddy, 1999; Rosebrock et al., 2007).

HopM1, another *P. syringae* T3E, mediates proteasomal degradation of Arabidopsis HopM interactor7 (AtMIN7), a plant adenosine diphosphate ribosylation factor-guanine nucleotide exchange factor, by a yet-unidentified ubiquitin ligase (Nomura et al., 2006). AtMIN7 degradation prevents proper vesicle trafficking and callose deposition, a hallmark of plant leaf PTI responses.

Finally, an alternative strategy deployed by plant pathogenic bacteria to subvert the host UPS is illustrated by the finding that *P. syringae* pv *syringae* secretes a small, nonribosomal peptide called SylA that can irreversible bind and inhibit the host proteasome. The absence of production of SylA strongly reduces the virulence of this strain on its host plant, indicating that inhibition of the proteasome is required for full pathogenicity (Groll et al., 2008).

Viral E3 Ubiquitin Ligases

F-box encoding genes have also been shown to be present in genomes of two plant viruses. The cell cycle

link (CLINK) F-box protein of the small DNA virus family has been shown to interact with plant SKP1-like and retinoblastoma-related proteins. Since CLINK is required for a normal level of viral DNA replication, it was proposed that destabilization of retinoblastoma-related proteins by a putative SCF^{CLINK} complex enables cell cycle progression and induces viral replication by releasing polymerase II inhibition. Nevertheless, stimulation of DNA replication by CLINK is independent of its functional F-box domain and direct evidence of SCF^{CLINK}-mediated protein ubiquitination and degradation is still lacking (Aronson et al., 2000; Lageix et al., 2007).

The P0 protein of polerovirus is an F-box protein that acts as a silencing suppressor and is important for viral proliferation (Pfeffer et al., 2002; Pazhouhandeh et al., 2006). Silencing suppression by P0 can be explained by the degradation of ARGONAUTE1 (AGO1), a key player in the RNA-induced silencing complex. Interestingly, P0-mediated destabilization of AGO1 does not seem to be dependent on the proteasome (Fig. 2; Baumberger et al., 2007; Bortolamiol et al., 2007).

In addition to its proteolytic activities, the UPS displays RNase activity associated with the α5 subunit and is able to degrade viral RNAs, suggesting that this activity may be part of a general antiviral defense pathway (Ballut et al., 2003; Dielen et al., 2011). Interestingly, a countereffect has been uncovered by the finding that the multifunctional HcPro viral protein (Helper component Protein), a potent suppressor of RNA silencing, associates with different 20S proteasomal subunits and interferes with the RNase activity of the 20S proteasome (Ballut et al., 2005; Jin et al., 2007; Dielen et al., 2011). Moreover, Arabidopsis mutants knocked out for each of the two *At-PAE* genes encoding the α5 subunit of the 20S proteasome were more susceptible to infection by *Lettuce mosaic potyvirus* (Dielen et al., 2011). In another study, a potato (*Solanum tuberosum*) RING-finger protein was found to physically interact with HcPro. Although no modification of HcPro accumulation could be detected in the presence of the RING protein, this finding suggests a mechanism to prevent HcPro-mediated counter defense of potyviruses (Guo et al., 2003).

Finally, the C4 viral protein from geminivirus induces the expression of Related to Kip1 ubiquitylation-Promoting Complex1 (RKP), a host RING-finger E3 ligase that seems to function as a regulator of the cell cycle. Indeed, RKP is able to target and contribute to the proteasomal degradation of the Arabidopsis cyclin-dependent kinase inhibitor Kip-Related Protein1 that functions in the G1-S transition of the cell cycle (Ren et al., 2008). Therefore, induction of RKP expression by the virus may account for the observed C4-induced abnormal cell division in Arabidopsis. It is hypothesized that this modification of the cell cycle may provide a more suitable environment for viral replication (Lai et al., 2009).

Oomycete and Fungal Effectors Interfering with the Plant UPS

Recent years have seen the advent of oomycete effector studies. Most of these parasites harbor several hundreds of effectors with probable overlapping functions. One of the few effectors having a drastic effect on *Phytophtora infestans* pathogenicity is Avr3A. This protein interacts with and stabilizes the plant U-box protein CMPG1. Avr3A suppresses the PTI associated with INF1-induced cell death in a process that requires CMPG1. By stabilizing CMPG1 and preventing its own degradation, Avr3A is hypothesized to additionally prevent the degradation of its targets and hence interfere with INF1-induced cell death (Bos et al., 2010). More recently, the structure of Avr3A from *Phytophtora capsici* was determined and identified a protein domain, other than the RxLR domain, responsible for the interaction with phosphatidylinositol monophosphate in vitro. This interaction between phosphatidylinositol monophosphates and the RxLR, originally described as being essential for the internalization of oomycete effectors (Kale et al., 2010), is now believed to be required for the accumulation of Avr3A inside the cell specifically during the suppression of INF1-induced cell death via interaction with CMPG1 (Yaeno et al., 2011).

To our knowledge, the only evidence that fungal effectors may interfere with their host UPS is the report that the *M. oryzae* AvrPiz-t interacts in yeast with four different plant proteins involved in the ubiquitination pathway although the implications of this finding are unknown (Liu et al., 2010).

Do Nematode and Insects Also Interfere with the Host UPS?

Intriguingly, the stylet-secreted protein cocktail of the cyst nematode *Heterodera schachtii* contains a new class of ubiquitin with an atypical C-terminal extension (Tytgat et al., 2004). A study to characterize the full stylet secretome identified two ubiquitin hydrolases, a ubiquitin-activating enzyme as well as an SKP1-like protein (Bellafiore et al., 2008). In a similar work to characterize the protein composition of the saliva injected by aphids into host cells, a putative ubiquitin-specific protease was identified (Carolan et al., 2011). It is therefore tempting to speculate that these stylet and salivary secreted proteins may interfere with the plant UPS during the infection process.

CONCLUSION AND FUTURE PERSPECTIVES

The past few years have witnessed the identification of a significant number of UPS-related components that modulate plant immune responses. These components appear to be involved in all aspects of plant immunity, from pathogen recognition to downstream signaling during both PTI and ETI responses. More

Table I. *Plant E3 ubiquitin ligase proteins and their function in the regulation of plant immunity*

E3 Ligase	Target[a]	Organism[b]	Function in Plant Immunity[c]	Reference
U-box				
MAC3A, 3B		*At*	Positive regulators of PTI and ETI to virulent and some avirulent *Pseudomonas* strains	Monaghan et al. (2009)
PUB12, 13	**FLS2**	*At*	Negative regulators of PTI to virulent and avirulent *Pseudomonas* strains	Lu et al. (2011)
PUB22, 23, 24		*At*	Negative regulators of PTI to virulent *Pseudomonas* and *Ha*	Trujillo et al. (2008)
PUB17		*At*	Positive regulator of ETI to *Pseudomonas*	Yang et al. (2006)
ACRE74/CMPG1		*Nt*/*Sl*	Positive regulator of ETI to *Cf*	González-Lamothe et al. (2006)
ACRE276		*Nt*,*Sl*	Positive regulator of ETI to *Cf*	Yang et al. (2006)
SPL11/PUB13		*Os*/*At*	Negative regulator of plant cell death	Yin et al. (2000); Zeng et al. (2004); Li et al. (2012)
RING				
ATL2		*At*/*Sl*	Overexpression leads to constitutive defense-related gene expression	Serrano and Guzmán (2004)
ATL9		*At*	Positive regulator of PTI to *Gc*	Berrocal-Lobo et al. (2010)
BAH1/NLA		*At*	Negative regulator of *Pseudomonas* infection-associated SA accumulation and defense	Yaeno and Iba (2008)
BOI1	**BOS1**	*At*	Negative regulator of cell death in response to *Bc*	Luo et al. (2010)
HUB1	MED21, **H2B**	*At*	Positive regulator of defense responses to *Bc* and *Ab*	Dhawan et al. (2009)
RIN2/RIN3	RPM1	*At*	Positive regulators of RPM1-mediated plant defense	Kawasaki et al. (2005)
RING1		*At*	Positive regulator of fumonisin B1-induced cell death	Lin et al. (2008)
RFP1	CABPR1	*Ca*	Overexpression in *At* confers disease susceptibility to virulent *Pseudomonas*	Hong et al. (2007)
RING1		*Ca*	Positive regulator of cell death against virulent and avirulent *Xanthomonas* strains	Lee et al. (2011)
BBI1		*Os*	Positive regulator of cell wall defense responses to blast fungus	Li et al. (2011)
RHC1		*Os*	Overexpression in *At* confers enhanced resistance to virulent *Pseudomonas* strains	Cheung et al. (2007)
XB3	XA21	*Os*	Positive regulator of PTI to *Xanthomonas*	Wang et al. (2006)
F-box				
CPR1/CPR30	SNC1, RPS2	*At*	Negative regulator of ETI to virulent and avirulent *Pseudomonas* strains	Gou et al. (2009, 2012); Cheng et al. (2011)
SON1		*At*	Positive regulator of SAR associated to virulent *Pseudomonas* and *Ha* infection	Kim and Delaney (2002)
ACIF1/ACRE189		*Nb* (*Sl*, *Nt*)	Positive regulator of ETI to *Cf*, TMV, and *Pseudomonas*	van den Burg et al. (2008)
DRF1		*Os*	Overexpression in *Nt* enhances resistance to TMV and *Pseudomonas* infection	Cao et al. (2008)

[a]In boldface, targets for which E3-mediated ubiquitination has been shown. [b]*At*, Arabidopsis; *Os*, rice; *Sl*, tomato; *Ca*, pepper; *Nb*, *N. benthamiana*; *Nt*, tobacco. [c]*Cf*, *C. fulvum*; *Bc*, *B. cinerea*; *Ab*, *Alternaria brassicicola*; *Gc*, *G. cichoracearum*; *Ha*, *H. arabidopsidis*.

particularly, numerous E3 ligase proteins have been identified as plant immunity regulators although, in most cases, their targets remain unknown (Table I). Future identification and characterization of these target proteins will undoubtedly provide new insights into the molecular mechanisms associated to plant defense. In addition, our knowledge about the contribution of monoubiquitination and other noncanonical forms of ubiquitination (and their outcomes) to plant immunity is still very poorly understood and needs further investigation. For example, in Arabidopsis, the RING E3 ligase HISTONE MONOUBIQUITINATION1 (HUB1) that monoubiquitinates histone H2B and interacts with MED21, a subunit of the Mediator complex that regulates the function of RNA polymerase II, has been involved in disease resistance against necrotrophic fungal pathogens (Dhawan et al., 2009). Investigation of additional outcomes of ubiquitination, other than proteasomal protein degradation, will thus provide a more complete picture of the varied regulatory roles associated to this posttranslational modification. Interestingly, deubiquitinating enzymes Arabidopsis Ubiquitin-Specific Protease12 (AtUBP12) and AtUBP13 have been found as negative regulators of plant defense, probably through stabilization of target substrates acting as immunity suppressors (Ewan et al., 2011). This finding warrants future research to determine how removal of ubiquitin by deubiquitinating enzymes determines the fate and activity of tagged substrates. Finally, the fact that most

microbes appear to have evolved a way to subvert the host UPS (1) underlines the importance of ubiquitination-related processes during the regulation of plant responses to pathogen attack and (2) provides a fascinating illustration of the degree of sophistication reached by pathogens in their attempt to colonize the host.

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