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REVIEW PAPER

The ubiquitin–proteasome system as a transcriptional regulator of plant immunity

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Abstract

The ubiquitin–proteasome system (UPS) has been shown to play vital roles in diverse plant developmental and stress responses. The UPS post-translationally modifies cellular proteins with the small molecule ubiquitin, resulting in their regulated degradation by the proteasome. Of particular importance is the role of the UPS in regulating hormone-responsive gene expression profiles, including those triggered by the immune hormone salicylic acid (SA). SA utilizes components of the UPS pathway to reprogram the transcriptome for establishment of local and systemic immunity. Emerging evidence has shown that SA induces the activity of Cullin-RING ligases (CRLs) that fuse chains of ubiquitin to downstream transcriptional regulators and consequently target them for degradation by the proteasome. Here we review how CRL-mediated degradation of transcriptional regulators may control SA-responsive immune gene expression programmes and discuss how the UPS can be modulated by both endogenous and foreign exogenous signals. The highlighted research findings paint a clear picture of the UPS as a central hub for immune activation as well as a battle ground for hijacking by pathogens.

Keywords: Cullin-RING, NPR1, plant immunity, salicylic acid, ubiquitin–proteasome system, WRKY transcription factors.

Introduction to the ubiquitin–proteasome system

Regulated degradation of short-lived or damaged proteins plays vital roles in cellular development and signalling across eukaryotes. The ubiquitin–proteasome system (UPS) is responsible for the selection, targeting, and proteolysis of specific substrates destined for degradation. UPS components are especially abundant in plants, suggesting that this system is a key hub for the regulation of numerous plant cell processes. Emerging evidence from plants and animals indicates that UPS components not only function merely to target substrates for degradation but are also critical transcriptional co-regulators that are indispensable for developmental and stress-responsive gene expression programmes (Santner and Estelle, 2009; Vierstra, 2009; Geng *et al.*, 2012; Kelley and Estelle, 2012).

Central to the functioning of the UPS is the post-translational modification of substrates by a single or polymeric chain of ubiquitin, a highly conserved small 8.5 kDa protein. Ubiquitin is covalently added to lysine residues of substrates in a multistep enzymatic cascade that involves E1 activating, E2 conjugating, and E3 ligase enzymes. First an E1 enzyme forms a high-energy thioester bond to an ubiquitin adduct, which is then transferred onto the active site cysteine residue of an E2 enzyme. The ubiquitin-loaded E2 enzyme then partners with an E3 ligase to transfer ubiquitin to a lysine residue of the target substrate. Reiterations of this reaction allow subsequent ubiquitin molecules to be similarly attached to internal lysine residues of the preceding ubiquitin, thus generating a chain of polyubiquitin on the substrate (Smalle and Vierstra, 2004; Komander and Rape, 2012). While chains can form by linking different lysine residues of ubiquitin, Lys48 linkage between four or more

ubiquitins exhibits high affinity for ubiquitin receptors of the proteasome, a large 2.5 MDa ATP-dependent chambered protease consisting of dozens of distinct subunits (Thrower *et al.*, 2000; Pickart and Cohen, 2004). The 19S regulatory cap of the proteasome is responsible for recognition of ubiquitinated substrates, the chaperone-assisted unfolding of substrates, and releasing polyubiquitin for recycling. Subsequently, unfolded substrates are threaded into the 20S particle of the proteasome, a barrel-shaped multicatalytic proteinase, where they are cleaved into peptides (Pickart and Cohen, 2004).

Compared with other eukaryotes, plant genomes often encode large numbers of UPS components, suggesting that the UPS plays important roles in diverse cellular processes. Recent years have clearly shown that the UPS contributes to the establishment of local and systemic immunity in plants. Comprehensive reviews on the role of ubiquitination in plant immunity are already available (Trujillo and Shirasu, 2010; Marino *et al.*, 2012; Duplan and Rivas, 2014; Furniss and Spoel, 2015), so here we provide a more focused update on our understanding of how selected components of the UPS function as transcriptional co-regulators of plant immune genes.

SA-responsive Cullin-RING ligases are transcriptional co-regulators

Amongst UPS components, E3 ligases are predominantly responsible for establishing selective UPS activity. E3 ligases specifically recognize and interact with their substrates, leading to their (poly)ubiquitination and, in the case of Lys48-linked ubiquitin chains, subsequent degradation by the proteasome. Plants contain large numbers of E3 ligases (e.g. the Arabidopsis genome encodes >1500) that are each predicted to target different substrate repertoires (Mazzucotelli *et al.*, 2006). Among these, the family of modular multisubunit Cullin-RING ligases (CRLs), which are predicted to form nearly 700 different E3 ligases, have been shown to be necessary for plant defence signalling by the immune hormones salicylic acid (SA) and jasmonic acid (JA).

Biotropic pathogen attack leads to rapid accumulation of SA, which acts as both a local and systemic signal for the induction of appropriate defences (Spoel and Dong, 2012). In incompatible plant–pathogen interactions, SA acts locally as an agonist of programmed cell death, which is thought to confine pathogens to a hostile environment and deprive them of further nutrients. Pathogen attack also leads to accumulation of SA in tissues adjacent or distant from the (attempted) infection site where it co-ordinates the reprogramming of ~2200 genes, including *pathogenesis-related* (*PR*) genes (Wang *et al.*, 2006). Genetic screens for SA-insensitive Arabidopsis mutants have repeatedly identified different *npr1* (*non-expressor of PR genes 1*) alleles (Cao *et al.*, 1994; Delaney *et al.*, 1995; Glazebrook *et al.*, 1996; Shah *et al.*, 1997). NPR1 encodes a transcriptional co-activator with two protein–protein interaction domains: an N-terminal BTB (Bric-à-brac Tramtrack, and Broad complex) domain and a more C-terminal Ankyrin repeat domain (Cao *et al.*, 1997; Ryals *et al.*, 1997; Tada *et al.*, 2008). In resting cells, conserved cysteine residues in and adjacent to the BTB domain form disulphide bonds, resulting in formation of a high molecular weight cytoplasmic NPR1 oligomer that is excluded from the nucleus (Kinkema *et al.*, 2000; Mou *et al.*, 2003). Accumulation of SA triggers changes in cellular redox potential that together with the thiol reductase action of thioredoxins lead to reduction of these disulphide bonds with subsequent release of NPR1 monomer that translocates to the nucleus (Kinkema *et al.*, 2000; Mou *et al.*, 2003; Tada *et al.*, 2008). Nuclear NPR1 monomer interacts with and transactivates transcription factors of the TGA and WRKY families that associate with SA-responsive gene promoters (Zhang *et al.*, 1999; Després *et al.*, 2000; Zhou *et al.*, 2000; Boyle *et al.*, 2009; Saleh *et al.*, 2015). Thus, NPR1 is thought to be a master co-activator of SA-responsive immune gene transcription.

Disrupting formation of the NPR1 oligomer by mutation of oxidant-sensitive Cys156 led to loss of long-term SA-induced resistance, indicating that the oligomer is indispensable for NPR1 homeostasis (Tada *et al.*, 2008). This effect was associated with a drastic decrease in NPR1 protein levels, suggesting that nuclear NPR1 protein is unstable. Indeed, pharmacological inhibition of the proteasome resulted in accumulation of NPR1 in the nucleus of resting cells and constitutive activation of its direct target genes (Spoel *et al.*, 2009). Co-immunoprecipitation experiments demonstrated that NPR1 associated with a nuclear CRL3 ligase (also known as BC3B for BTB/Cullin3/BTB). Genetic perturbation of CRL3 by mutation of the Cullin 3 subunit, or by mutating the COP9 signalosome that regulates the stability and activity of Cullin proteins (Petroski and Deshaies, 2005), stabilized NPR1 protein and was associated with constitutive expression of its target genes. This suggests that CRL3-mediated degradation of NPR1 functions to maintain SA-responsive immune gene expression in a latent state, thereby preventing onset of autoimmunity (Fig. 1).

Despite being necessary for the SA-responsive activation of target genes, paradoxically SA-induced NPR1 was also a substrate for CRL3-mediated ubiquitination and proteasomal degradation (Spoel *et al.*, 2009). Mutation of CRL3 or an NPR1 phospho-site responsible for recruitment of NPR1 to CRL3 led to reduced SA-responsive gene expression and impaired disease resistance. These findings suggest that instability of NPR1 appeared to be necessary for full activation of its target genes. As we have proposed previously (Spoel *et al.*, 2009; Furniss and Spoel, 2015; Skelly *et al.*, 2016), this may be due to gene expression requiring continuous delivery of fresh transcriptionally competent NPR1 to active gene promoters (Fig. 1). Although most transcriptional regulators are stable proteins, selected eukaryotic transcriptional activators have been found to exhibit an instability similar to NPR1. These activators often contain overlapping sequences that act as transactivation domains and degradation motifs (Salghetti *et al.*, 2000). NPR1 was found to form a transactivating transcriptional complex with TGA2 transcription factors, which required core residues of the BTB domain as well as oxidation of two C-terminal cysteine residues (Rochon *et al.*, 2006). Although these transactivation domains differ from the N-terminal phosphorylation sites that are necessary for recruitment of NPR1 to CRL3 (Spoel *et al.*, 2009), it is plausible that lysine ubiquitination occurs in or near these domains.

In rice, the *Oryza sativa* WRKY45 transcriptional activator exhibits overlap between trans-activating and

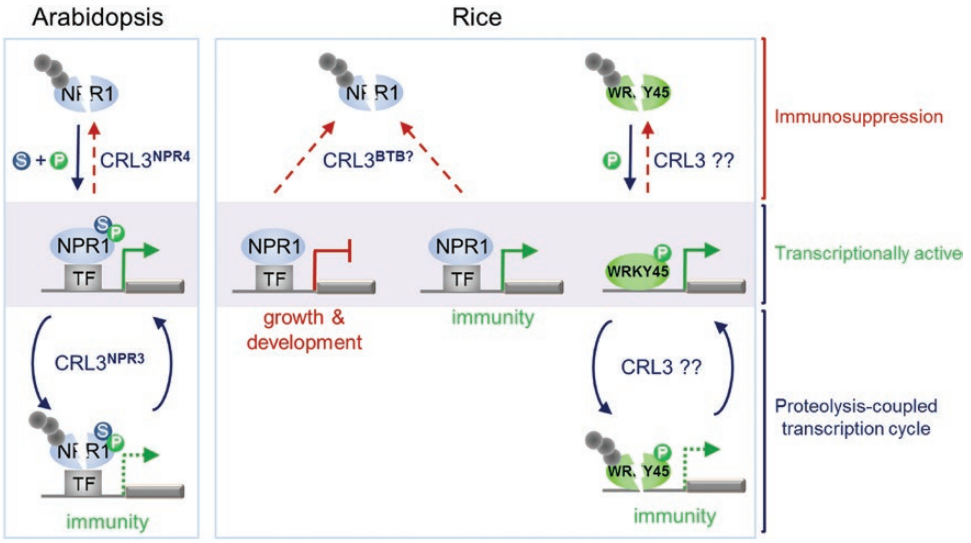

Fig. 1. CRL3-mediated transcriptional reprogramming in Arabidopsis and rice. In Arabidopsis (left panel), the stability of the transcriptional co-activator NPR1 is controlled by different CRL3 ligases. In unchallenged cells, NPR1 is polyubiquitinated (grey circles) by $CRL3^{NPR4}$ ligases to prevent autoimmunity (i.e. immunosuppression). Upon pathogen challenge, SA induces the SUMOylation (S, blue cirlces) and phosphorylation (P, green circles) of NPR1, allowing transcriptionally active NPR1 to undergo a transcription-coupled proteolysis cycle that stimulates the expression of immune genes. In rice (right panel), immunosuppression is accomplished by CRL3-mediated ubiquitination and degradation of OsNPR1 as well as by degradation of OsWRKY45 which may also involve a CRL3 ligase. Upon pathogen challenge, OsNPR1 activates immune genes but also suppresses genes involved in growth and development. Additionally, pathogen challenge activates OsWRKY45, resulting in its phosphorylation and subsequent degradation in a proteolysis-coupled transcription cycle that is hypothesized to involve a CRL3 ligase. Note that the transcriptionally competent or active state of all transcription (co) regulators is represented by a shaded box.

proteolysis-targeting sequences. OsWRKY45 performs a very similar function to Arabidopsis NPR1, as it is responsible for SA-responsive transcriptional reprogramming and establishment of resistance against bacterial and fungal pathogens (Shimono *et al.*, 2007, 2012; Nakayama *et al.*, 2013). Pharmacological treatment with proteasome inhibitors resulted in accumulation of ubiquitinated OsWRKY45 and blocked SA-induced transcriptional activation of its target genes (Matsushita *et al.*, 2013). Importantly, C-terminal sequences in OsWRKY45 were necessary for both its transcriptional activity and UPS-dependent degradation. Thus, SA-responsive gene expression in higher plants may be dependent on transcriptional regulators harbouring sequences that function as both trans-activating domains and UPS targeting signals (Fig. 1).

While it remains unknown which E3 ligase is responsible for targeting OsWRKY45 for proteasome-dependent degradation, a CRL3 ligase has been implicated in SA-dependent immunity in rice. Studies on *Cullin 3a* (*OsCUL3a*) revealed that it interacted with RING-BOX1 (RBX1) and RBX2 to constitute the core of CRL3 (Liu *et al.*, 2017). Genetic analyses showed that *oscul3a* mutants displayed typical symptoms of autoimmune activation, suggesting that CRL3 functions as an immunosuppressant in rice. Similar to Arabidopsis NPR1, OsWRKY45 is continuously degraded by the UPS in resting cells, and failure to clear this activator results in autoimmune phenotypes reminiscent of *oscul3a* (Matsushita *et al.*, 2013). Thus, it is plausible that in analogy to Arabidopsis, CRL3 also targets OsWRKY45 for proteasome-mediated degradation in rice (Fig. 1).

OsCUL3 was found to associate physically with the rice homologue of NPR1, known as OsNPR1 or NH1, which also functions in an SA-responsive immune signalling pathway. Cycloheximide and proteasome inhibition assays established that OsCUL3 is necessary for the proteasome-dependent degradation of OsNPR1, indicating that OsNPR1 is also a substrate of CRL3 (Liu *et al.*, 2017). Rice CRL3 has the potential to influence a large transcriptional immune programme, as accumulation of OsNPR1 protein in *oscul3a* mutants was associated with activation of *PR* genes. This is in agreement with previous reports demonstrating that overexpression of OsNPR1 resulted in constitutive activation of immune genes and resistance to bacterial blight (Chern *et al.*, 2001, 2005; Yuan *et al.*, 2007). However, genome-wide transcript profiling of *OsNPR1* knock-down lines showed that its function as an activator of immune genes was relatively modest in comparison with its role in transcriptional suppression. OsNPR1 directly or indirectly down-regulated the expression of genes involved in photosynthesis and in chloroplast translation and transcription, suggesting that it plays an important role in resource reallocation during establishment of immunity (Sugano *et al.*, 2010). How CRL3-mediated ubiquitination of OsNPR1 affects suppression of these growth- and development-related genes currently remains unknown. Considering that CRL3 controls the cellular levels of OsNPR1, it is expected that CRL3 plays an important role in limiting the suppressive effects of NPR1, thereby managing the balance between defence and growth (Fig. 1).

Composition and potential structure of CRL3 ligases in SA signalling

To recruit substrates, CRL3 ligases utilize specific substrate adaptors that typically contain a BTB domain and one or

more additional protein–protein interaction domains. The BTB domain and adjacent sequences directly interact with CUL3 (Stogios *et al.*, 2005; Zhuang *et al.*, 2009; Canning *et al.*, 2013), while the additional protein–protein interaction domain binds to the substrate. Intriguingly, the *NPR* protein family appears to have all the necessary features to function as substrate adaptors for CUL3. In Arabidopsis, this family consists of *NPR1* and five *NPR1*-like genes, namely *NPR1-like 2* (*NPR2*), *NPR3*, *NPR4*, *BLADE-ON-PETIOLE2* (*BOP2*; also named *NPR5*), and *BOP1* (also named *NPR6*). Each of the NPR1 proteins contains a conserved BTB and Ankyrin repeat domain. Whereas BOP1 and BOP2 contribute to leaf development and JA-mediated immune signalling (Hepworth *et al.*, 2005; Canet *et al.*, 2012), NPR1–NPR4 have all been implicated in SA-dependent immune signalling (Cao *et al.*, 1997; Liu *et al.*, 2005; Zhang *et al.*, 2006; Canet *et al.*, 2010; Fu *et al.*, 2012). While little is known about the role of NPR2 in defence signalling, it was demonstrated that genetically NPR2 contributes to SA perception in *npr1* null mutants (Canet *et al.* 2010). Recent work indicated that recruitment of NPR1 to CRL3 was dependent on both NPR3 and NPR4, suggesting that these proteins recruit NPR1 for ubiquitination by a $CRL3^{NPR3/4}$ ligase. This ligase was found to be necessary for establishment of SA-dependent systemic acquired resistance and pathogen effector-induced programmed cell death responses (Fu *et al.*, 2012). Although it remains unclear how and if NPR3 and NPR4 regulate the transcriptional activity of NPR1, these results clearly indicate the potential for NPR protein family members to provide specificity to CRL3 ligases in plant immunity.

Computational and protein crystallization data have shown that CRL3 ligases are dimeric (Stogios *et al.*, 2007; Zhuang *et al.*, 2009; Zimmerman *et al.*, 2010). Dimerization is driven by tight electrostatic interactions between BTB domains of two substrate adaptors, allowing the binding of two CUL3 subunits. Rather than recruiting two substrates, CRL3 dimerization has been suggested to improve the efficiency with which a single substrate molecule is ubiquitinated. It is thought that the dimer constrains the mobility of the substrate, thereby improving the rate of ubiquitination on target lysines (McMahon *et al.*, 2006). Because self-association was found to be a general feature of many CRL3 ligases (McMahon *et al.*, 2006), it is likely that NPR3 and NPR4 allow CRL3 in plants to form a dimeric complex that recruits NPR1 for ubiquitination. Current work in our laboratory is investigating if CRL3 forms homo- or heterodimers. Although heterodimers are not documented as a feature of BTB-containing CRL3 substrate adaptors, it has been demonstrated for two hetero-oligomerizing F-box proteins, Pop1p and Pop2p, which are part of a Skip-Cullin-F-box (SCF, also known as CRL1) ligase in fission yeast (Seibert *et al.*, 2002). While heterodimeric $SCF^{Pop1p–Pop2p}$ targets the cyclin-dependent kinase inhibitor Rum1p for degradation, homodimeric SCF^{Pop1p} and SCF^{Pop2p} complexes probably have different substrate preferences. Thus heterodimer or even hetero-oligomer formation between different NPR proteins potentially increases combinatorial diversity in substrate preference that could extend well beyond NPR1 as the sole substrate.

Co-activator turnover is modulated by endogenous and exogenous signals

The destructive nature of the UPS must be tightly controlled to ensure appropriate levels of substrate degradation. A major regulatory checkpoint is the selective recruitment of substrates to E3 ligases. Diverse cellular signals including post-translational modifications mark substrates for recruitment to E3 ligases. Activation of nuclear NPR1 and its recruitment to CRL3 was recently shown to involve complex interplay between SUMOylation and (de)phosphorylation (Saleh *et al.*, 2015). SA-induced dephosphorylation of Ser55/59 was prerequisite for modification of NPR1 by SUMO3. NPR1 SUMOylation was proposed to regulate positional interactions with its target promoters through differential association with transcription factor partners. Whereas unmodified NPR1 interacted with the transcriptional repressor WRKY70, SUMOylated NPR1 preferentially associated with the transcriptional activator TGA3. Importantly, SUMOylation itself or the resulting switch in transcriptional partner (i.e. from WRKY70 to TGA3) was required for subsequent phosphorylation of Ser11/15 (Saleh *et al.*, 2015). Ser11/15 phosphorylation was in turn necessary for recruitment of NPR1 to CRL3, resulting in its ubiquitination and turnover by the proteasome (Fig. 1) (Spoel *et al.*, 2009). Although the exact residues remain unknown, OsWRKY45 in rice was also found to be phosphorylated. Interestingly, the phosphorylated form was highly responsive to proteasome inhibition, suggesting that site-specific phosphorylation of OsWRKY45 may also be required for its UPS-dependent degradation (Fig. 1) (Matsushita *et al.*, 2013). Thus, extensive interplay between diverse post-translational signals regulates the stability and associated activity of SA-responsive transcriptional (co)activators (Skelly *et al.*, 2016).

Recruitment of substrates to E3 ligases is not only regulated by post-translational control mechanisms, but it may also be facilitated or inhibited by small molecules. While this has driven the design of synthetic molecules for human medicine (Zheng and Shabek, 2017), in plants several major developmental and stress signalling pathways naturally utilize E3 ligases as receptors for small-molecule hormones. This was first discovered for the plant developmental hormone auxin, which promotes the recruitment of a family of transcriptional repressors to an SCF^{TIR1} ubiquitin ligase. Structural biology approaches have shown that auxin acts as 'molecular glue' by enhancing protein–protein interactions between the SCF F-box subunit TIR1 and transcriptional repressors. Consequently, auxin perception at or near the chromatin relieves transcriptional suppression by SCF^{TIR1}-mediated ubiquitination and degradation of repressors (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Santner and Estelle, 2009). Other hormones, such as JA-isoleucine, are perceived similarly by different SCF ligase–substrate complexes, indicating that hormone perception by E3 ligases is a key mechanism for direct transcriptional regulation (Kelley and Estelle, 2012). Intriguingly, SA is also perceived by the CRL3 substrate adaptors NPR3 and NPR4, suggesting that hormone perception may be a common mechanism of perception by CRL ubiquitin ligases. Distinct from other hormones, however, SA also regulates how and when

NPR3 and NPR4 interact with NPR1. This is a consequence of the vastly different affinities NPR3 and NPR4 have for SA. Whereas NPR3 has a relatively low affinity for SA, NPR4 displays much higher affinity. Moreover, SA binding has opposing effects on substrate recruitment. The result is that elevated levels of SA disrupt NPR1–NPR4 interactions, but promote NPR1–NPR3 interactions (Fu *et al.*, 2012). Thus, in the absence of pathogen threat when low levels of SA are present, the $CRL3^{NPR4}$ ubiquitin ligase complex is thought to suppress NPR1 monomer levels to prevent autoimmunity. Conversely, when cellular levels of SA increase following pathogen attack, NPR1 recruitment is switched from $CRL3^{NPR4}$ to $CRL3^{NPR3}$, which is necessary for local and systemic immune responses (Fu *et al.*, 2012; Furniss and Spoel, 2015). Although details of the mechanisms by which SA regulates $CRL3^{NPR3/NPR4}$ remain largely unknown, the clear analogy to other CRL-dependent signalling pathways demonstrates that SA is a key small molecule involved directly in the regulation of NPR1 stability and therefore probably also NPR1 transcriptional potency.

Endogenous signals originating from hormone signalling pathways may also modulate CRL3-mediated ubiquitination and degradation of NPR1 co-activator. The developmental hormone abscisic acid (ABA) interacts antagonistically with SA-dependent immune signalling. Some pathogens hijack this antagonism by inducing apparent increases in ABA biosynthesis, thereby inhibiting signalling steps both up- and downstream of SA accumulation (de Torres-Zabala *et al.*, 2007; Mohr and Cahill, 2007; Yasuda *et al.*, 2008). A recent report investigated how ABA might impact SA signalling by examining NPR1 stability (Ding *et al.*, 2016). It was found that treatment with ABA strongly reduced the cellular level of NPR1 in a $CRL3^{NPR3/NPR4}$- and proteasome-dependent manner, suggesting that in unchallenged cells ABA antagonized SA signalling by destabilizing NPR1. However, by changing the timing of pharmacological applications of SA and ABA, it was found that ABA reduces NPR1 protein levels only if ABA treatment preceded SA treatment. These data suggest that ABA has less control over the stability of SA-induced NPR1. Indeed, phosphorylation of Ser11/15, which is necessary for SA-induced NPR1 degradation, appeared to block ABA-induced NPR1 instability. Nonetheless, ABA treatment could strongly reduce SA-induced *PR-1* gene expression, implying that antagonism between SA and ABA is more complex than can be observed by examining NPR1 protein levels at a single time point. By temporally surveying NPR1 protein levels during infection by the virulent bacterial pathogen *Pseudomonas syringae*, the authors revealed that SA and ABA accumulate sequentially, which may allow a switch from SA-induced NPR1 protein degradation to ABA-induced turnover (Ding *et al.*, 2016). The functional outcome of switching between mechanisms of NPR1 degradation remains unclear, but ABA-induced degradation of NPR1 during later stages of the immune response might be necessary for full-scale induction of NPR1-dependent target genes. How ABA recruits NPR1 for degradation by $CRL3^{NPR3/4}$ also remains unknown, but it may well involve phosphorylation of NPR1 by members of the SNF1-related protein kinase (SnRK) family. SnRK members have been implicated in ABA signalling by promoting the transcriptional activity of ABA-responsive co-activators. More recently, SnRK2.8 was found to phosphorylate NPR1 at Ser589 and possibly Thr373, allowing its nuclear translocation in tissues distal from the site of infection (Lee *et al.*, 2015). It is plausible that co-ordination between ABA- and SA-induced phosphorylation events orchestrates diverse pathways of NPR1 ubiquitination and degradation, each with distinct transcriptional outputs.

In addition to endogenous inputs, exogenous signals may also modulate transcriptional co-activator turnover. Curiously, pathogen effectors from a variety of plant pathogens have been shown to interfere with components of the host UPS machinery. Some effectors have been found to inhibit the activity of immune-related E3 ligases or enhance their stability, while others such as *P. syringae* avrPtoB mimic RING- and U-box-type E3 ligases and target host pathogen recognition receptors for degradation (Duplan and Rivas, 2014). More recently it was shown that the infection strategy of *P. syringae* includes inhibition of host proteasome activity in a type III secretion-dependent manner (Üstün *et al.*, 2016). A screen for secreted effectors uncovered several proteins, including HopM1, with proteasome inhibitor activity. Co-immunoprecipitation experiments showed that HopM1 complexed with a variety of E3 ligases and proteasome subunits, demonstrating that it directly targets the UPS (Üstün *et al.*, 2016). Other pathogen effectors have also been identified to target the proteasome directly. The *Xanthomonas campestris* effector protein XopJ was found to suppress host proteasome activity by degrading the proteasomal AAA-ATPase subunit RPT6 (Üstün *et al.*, 2013; Üstün and Börnke, 2015). The ATPase activity of RPT6 is thought to be required for the unfolding of substrates prior to their insertion into the 20S catalytic barrel. RPT6 contains Walker A and Walker B motifs that are essential for its ability to bind and hydrolyse ATP, respectively. Interestingly, it was shown that mutation of the Walker A motif abolished interaction with XopJ, whereas mutation of the Walker B motif prevented XopJ-mediated proteolysis of RPT6 (Üstün and Börnke, 2015). These findings suggest that only ATP-bound RPT6 is recognized by XopJ and that XopJ may mimic host substrates intended for proteasomal degradation. Importantly, XopJ-mediated proteolysis of RPT6 was linked to increased accumulation of ubiquitinated NPR1 and decreased turnover of this transcriptional co-activator, probably preventing full-scale activation of SA-responsive immune genes (Spoel *et al.*, 2009; Üstün and Börnke, 2015).

Because of its critical role in the activation of SA-responsive immune genes, it has long been speculated that pathogen effectors also directly target NPR1 and suppress its transcriptional co-activator activity. Indeed, recent work identified the *P. syringae* effector avrPtoB, a U-box type E3 ligase, as an interactor of NPR1 (Chen *et al.*, 2017). Curiously, SA enhanced interaction between avrPtoB and NPR1, leading to NPR1 ubiquitination and subsequent proteasomal degradation. Unlike immune-induced NPR1 ubiquitination and turnover, avrPtoB-induced degradation of NPR1 negatively affected SA-responsive gene expression and immunity (Chen *et al.*, 2017). This suggests either that avrPtoB ubiquitinates distinct lysine residues in NPR1 as compared with CRL3 or that it targets NPR1 for ubiquitination prior to its recruitment

to CRL3. As SA promotes interaction between avrPtoB and NPR1, it is plausible that avrPtoB titrates NPR1 away from SA-mediated binding to the CRL3 substrate adaptor NPR3.

Degradation of other SA-responsive transcriptional immune regulators

The UPS may control SA-responsive gene expression in ways that go well beyond regulating the stability of master (co)activators such as NPR1 and OsWRKY45. SA-responsive gene expression is modulated by many other transcriptional activators and repressors from the TGA, WRKY, and NIMIN or OsNRR families, many of which physically interact with NPR1 and the CRL3 substrate adaptors NPR3 and NPR4 (Weigel *et al.*, 2001; Liu *et al.*, 2005; Zhang *et al.*, 2006; Shi *et al.*, 2013; Chern *et al.*, 2014; Saleh *et al.*, 2015). Interestingly, some of these transcriptional regulators or their close relatives have been reported to exhibit UPS-dependent instability. TGA1 and TGA3 transcriptional activators have been shown to interact with NPR proteins, and their protein levels are regulated by post-transcriptional mechanisms. Although their protein levels appeared to be controlled developmentally, inhibition of the proteasome resulted in accumulation of TGA3 in the nucleus (Pontier *et al.*, 2002). This suggests that TGA3 is unstable and its degradation could be managed by SA. Because TGA3 interacts with NPR3 and NPR4 (Zhang *et al.*, 2006), it is plausible that TGA3 is regulated by an SA-induced $CRL3^{NPR3}$ or $CRL3^{NPR4}$. Alternatively, TGA3 could be targeted for degradation indirectly through its interaction with NPR1 via concurrent ubiquitination and degradation. Concurrent ubiquitination and degradation of multiple physically associated substrates has already been reported for a CRL3 ligase involved in light signalling (Ni *et al.*, 2014). In addition to TGA transcription factors, WRKY transcription factors regulate SA-responsive gene expression both positively and negatively. While (in)stability of the wider WRKY protein family has not yet been examined, some WRKY proteins such as the above-discussed OsWRKY45 have been found to be subject to UPS-dependent degradation. In Arabidopsis, WRKY53 was found to be a substrate of HECT domain-containing Ubiquitin Protein Ligase 5 (UPL5) during leaf senescence (Miao and Zentgraf, 2010). Notably WRKY53 is also an activator of SA-responsive immune genes (Wang *et al.*, 2006), implying the possibility that control of WRKY53 protein levels by the UPS also impacts SA-dependent gene expression. In Chinese wild grapevine *Vitis pseudoreticulata*, WRKY11 (VpWRKY11) was targeted for ubiquitination and degradation by the RING E3 ligase *Erysiphe necator*-induced RING finger protein 1 (EIRP1), which was necessary for resistance to a variety of different pathogens, but specific effects on SA-responsive gene expression remained unclear. (Yu *et al.*, 2013). Finally, in Arabidopsis, Signal Responsive 1 (SR1), a Ca^{2+}/calmodulin-binding transcription factor, was found to be controlled by ubiquitination and degradation. SR1 binds to and suppresses the promoter of *Enhanced Disease Susceptibility 1* (*EDS1*), a gene involved in the biosynthesis of SA. Consequently, mutant *sr1-1* plants exhibit increased transcript levels of *EDS1* as well as other SA biosynthesis genes, and accumulate elevated levels of SA (Du *et al.*, 2009). Recent work demonstrated that SR1 is recruited to a CRL3 ligase for ubiquitination and proteasome-mediated degradation. Interestingly, SR1 is recruited to CRL3 by SR1 Interacting Protein 1 (SR1IP1), a protein containing both BTB and non-phototrophic hypocotyl 3 (NPH3) protein–protein interaction domains, which are typical characteristics of a CRL3 adaptor (Stogios *et al.*, 2005; Zhang *et al.*, 2014). SR1IP1 was shown to function as a positive regulator of SA-mediated defence responses by removing the transcriptional repressor SR1. Taken together with knowledge gained on $CRL3^{NPR}$ ligases, these findings imply the exciting possibility that CRL3 dynamically switches between different substrate adaptors to recruit distinct transcriptional (co)regulators for ubiquitination and degradation.

Perspectives

The complex roles of the UPS in regulating eukaryotic gene expression have been an intense field of study for some time now (Collins and Tansey, 2006; Geng *et al.*, 2012). Involvement of the UPS in plant immune transcriptional reprogramming is now well established, but the complexity is only just being uncovered. Aside from regulating SA-mediated immunity, the UPS is also vital in the control of JA-responsive gene expression during development and immunity. JA facilitates the molecular association between SCF^{COI1} ligase and its substrates, JAZ transcriptional co-repressors. SCF^{COI1}-mediated degradation of JAZ co-repressors releases numerous transcription factors from suppression and leads to the activation of, amongst others, defence responses against necrotrophic pathogens and insects, a topic extensively reviewed elsewhere (Goossens *et al.*, 2016; Zhang *et al.*, 2017). Interestingly, under many circumstances the SA and JA signals are antagonistic. SA exerts its antagonisms through the function of NPR1, which was uncovered as a potent suppressor of JA-responsive gene expression (Spoel *et al.*, 2003). It remains unclear if there are any spatial chromatin interactions between SA-responsive $CRL3^{NPR}$ and JA-responsive SCF^{COI1} ligases, but evidence suggests that SA and NPR1 suppress JA signalling further downstream. Indeed, activation of SA signalling failed to interfere with SCF^{COI1}-mediated degradation of JAZ co-repressors. Instead, SA strongly reduced protein levels of the JA-responsive transcriptional activator ORA59, which functions downstream of SCF^{COI1} (Van der Does *et al.*, 2013). Future research should reveal if this negative effect of SA on ORA59 protein levels is mediated by an SA-induced $CRL3^{NPR}$ ligase. This is highly plausible as a recent report demonstrated that $CRL3^{NPR3/NPR4}$ may also target transcriptional components of JA signalling during pathogen effector-triggered immunity (Liu *et al.*, 2016). Unlike local immune responses to virulent pathogens, immunity triggered by the recognition of pathogen effectors is not associated with antagonisms between the SA and JA signals (Spoel *et al.*, 2007). It was found that antagonism is avoided through $CRL3^{NPR3/NPR4}$-mediated ubiquitination and removal of JAZ co-repressors, allowing activation of JA-responsive genes in a cellular environment of active SA signalling. Curiously, pharmacological

application of SA was insufficient to induce degradation of JAZ co-repressors by $CRL3^{NPR3/NPR4}$, suggesting that effector recognition triggers additional signalling pathways that activate or recruit this E3 ligase. Taken together, these findings highlight the complexity of CRL functions and substrate interactions in transcriptional reprogramming during establishment of immunity.

Beyond E3 ligases, the proteasome itself may also play important roles in the regulation of gene expression programmes. In yeast and human cells, the proteasome has been found to associate physically with chromatin and regulate the expression of thousands of genes (Collins and Tansey, 2006; Geng *et al.*, 2012). Our understanding of how proteasomes are recruited to the chromatin sites where they are most needed is still in its infancy, but may be dependent on both E3 ligases and their substrates. In this respect, it is interesting to note that many E3 ligases interact with the 19S proteasome particle, suggesting that they might directly hand over ubiquitinated substrates for degradation (Schmidt *et al.*, 2005). Moreover, the proteasome may have resident E3 ligases that further modify substrates before their degradation (Schmidt *et al.*, 2005; Crosas *et al.*, 2006), indicating that further signalling complexity is achieved at the proteasome itself. Thus, to gain full appreciation of how the UPS controls transcriptional reprogramming in plant immunity, the future challenge is to uncover post-translational regulation and substrate repertoires of E3 ligases and the proteasome itself across different interconnected immune signalling pathways.

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