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# The ubiquitin-proteasome system as a transcriptional regulator of plant immunity

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36 **Highlights**
37 Activation of plant immunity is associated with dramatic changes in gene expression. Here
38 we discuss diverse roles of the ubiquitin-proteasome system as a transcriptional regulator of
39 immune genes.
40
41
42 **Abstract**
43 The ubiquitin-proteasome system (UPS) has been shown to play vital roles in diverse plant
44 developmental and stress responses. The UPS post-translationally modifies cellular proteins
45 with the small molecule ubiquitin, resulting in their regulated degradation by the proteasome.
46 Of particular importance is the role of the UPS in regulating hormone-responsive gene
47 expression profiles, including those triggered by the immune hormone salicylic acid (SA). SA
48 utilises components of the UPS pathway to reprogram the transcriptome for establishment of
49 local and systemic immunity. Emerging evidence has shown that SA induces the activity of
50 Cullin-RING ligases (CRLs) that fuse chains of ubiquitin to downstream transcriptional
51 regulators and consequently target them for degradation by the proteasome. Here we review
52 how CRL-mediated degradation of transcriptional regulators may control SA-responsive
53 immune gene expression programmes and discuss how the UPS can be modulated by both
54 endogenous and foreign exogenous signals. The highlighted research findings paint a clear
55 picture of the UPS as a central hub for immune activation as well as a battle ground for
56 hijacking by pathogens.
57

58 **Introduction to the Ubiquitin-Proteasome System**

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

68 Central to the functioning of the UPS is the post-translational modification of
69 substrates by a single or polymeric chain of ubiquitin, a highly conserved small 8.5 kDa
70 protein. Ubiquitin is covalently added to lysine (Lys) residues of substrates in a multi-step
71 enzymatic cascade that involves E1 activating, E2 conjugating and E3 ligase enzymes. First
72 an E1 enzyme forms a high-energy thioester bond to an ubiquitin adduct, which is then
73 transferred onto the active site cysteine (Cys) residue of an E2 enzyme. The ubiquitin-loaded
74 E2 enzyme then partners with an E3 ligase to transfer ubiquitin to a Lys residue of the target
75 substrate. Reiterations of this reaction allow subsequent ubiquitin molecules to be similarly
76 attached to internal Lys residues of the preceding ubiquitin, thus generating a chain of
77 polyubiquitin on the substrate (Komander and Rape, 2012; Smalle and Vierstra, 2004).
78 While chains can form by linking different Lys residues of ubiquitin, Lys48 linkage between
79 four or more ubiquitins exhibits high affinity for ubiquitin receptors of the proteasome, a large
80 2.5 MDa ATP-dependent chambered protease consisting of dozens of distinct subunits
81 (Pickart and Cohen, 2004; Thrower *et al.*, 2000). The 19S regulatory cap of the proteasome
82 is responsible for recognition of ubiquitinated substrates, the chaperone-assisted unfolding
83 of substrates, and releasing polyubiquitin for recycling. Subsequently unfolded substrates
84 are threaded into the 20S particle of the proteasome, a barrel-shaped multi-catalytic
85 proteinase, where they are cleaved into peptides (Pickart and Cohen, 2004).

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

94

**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 recognise and interact with their substrates, leading to their (poly)ubiquitination and, in case of Lys48-linked ubiquitin chains, subsequent degradation by the proteasome. Plants contain large numbers of E3 ligases (*e.g.* the *Arabidopsis* genome encodes for >1,500) that are predicted to each target different substrate repertoires (Mazzucotelli *et al.*, 2006). Among these the family of modular multi-subunit Cullin-RING Ligases (CRL), 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 coordinates the reprogramming of ~2,200 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 coactivator 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 Cys 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 trans-activates transcription factors of the TGA and WRKY families that associate with SA-responsive gene promoters (Boyle *et al.*, 2009; Després *et al.*, 2000; Saleh *et al.*, 2015; Zhang *et al.*, 1999; Zhou *et al.*, 2000). Thus, NPR1 is thought to be a master coactivator 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 the oligomer is indispensable for NPR1 homeostasis (Tada *et al.*, 2008). This effect was associated with a drastic decrease in NPR1 protein levels, suggesting nuclear NPR1 protein is unstable. Indeed, pharmacological

132 inhibition of the proteasome resulted in accumulation of NPR1 in the nucleus of resting cells
133 and constitutive activation of its direct target genes (Spoel *et al.*, 2009). Co-
134 immunoprecipitation experiments demonstrated that NPR1 associated with a nuclear CRL3
135 ligase (also known as BC3B for BTB/Cullin3/BTB). Genetic perturbation of CRL3 by mutation
136 of the Cullin 3 subunit or by mutating the COP9 signalosome that regulates the stability and
137 activity of Cullin proteins (Petroski and Deshaies, 2005), stabilised NPR1 protein and was
138 associated with constitutive expression of its target genes. This suggests that CRL3-
139 mediated degradation of NPR1 functions to maintain SA-responsive immune gene
140 expression in a latent state, thereby preventing onset of autoimmunity (Figure 1).

141 Despite being necessary for the SA-responsive activation of target genes,
142 paradoxically SA-induced NPR1 was also a substrate for CRL3-mediated ubiquitination and
143 proteasomal degradation (Spoel *et al.*, 2009). Mutation of CRL3 or an NPR1 phospho-site
144 responsible for recruitment of NPR1 to CRL3 led to reduced SA-responsive gene expression
145 and impaired disease resistance. These findings suggest that instability of NPR1 appeared
146 to be necessary for full activation of its target genes. As we have proposed previously
147 (Furniss and Spoel, 2015; Skelly *et al.*, 2016; Spoel *et al.*, 2009), this may be due to gene
148 expression requiring continuous delivery of fresh transcriptionally competent NPR1 to active
149 gene promoters (Figure 1). Although most transcriptional regulators are stable proteins,
150 selected eukaryotic transcriptional regulators have been found to exhibit a similar instability
151 as NPR1. These regulators often contain overlapping sequences that act as transactivation
152 domains and degradation motifs (Salghetti *et al.*, 2000). NPR1 was found to form a trans-
153 activating transcriptional complex with TGA2 transcription factors, which required core
154 residues of the BTB domain as well as oxidation of two C-terminal cysteine residues
155 (Rochon *et al.*, 2006). Although these transactivation domains differ from the N-terminal
156 phosphorylation sites that are necessary for recruitment of NPR1 to CRL3 (Spoel *et al.*,
157 2009), it is plausible that Lys ubiquitination occurs in or near these domains.

158 In rice, the *Oryza sativa* WRKY45 transcriptional activator exhibits overlap between
159 trans-activating and proteolysis targeting sequences. OsWRKY45 performs a very similar
160 function as Arabidopsis NPR1, as it is responsible for SA-responsive transcriptional
161 reprogramming and establishment of resistance against bacterial and fungal pathogens
162 (Nakayama *et al.*, 2013; Shimono *et al.*, 2012; Shimono *et al.*, 2007). Pharmacological
163 treatment with proteasome inhibitors resulted in accumulation of ubiquitinated OsWRKY45
164 and blocked SA-induced transcriptional activation of its target genes (Matsushita *et al.*,
165 2013). Importantly, C-terminal sequences in OsWRKY45 were necessary for both its
166 transcriptional activity as well as UPS-dependent degradation. Thus, SA-responsive gene
167 expression in higher plants may be dependent on transcriptional regulators that harbour

168 sequences that function as both trans-activating domains and UPS targeting signals (Figure
169 1).

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

180 OsCUL3 was found to physically associate with the rice homologue of NPR1, known
181 as OsNPR1 or NH1, which also functions in an SA-responsive immune signalling pathway.
182 Cycloheximide and proteasome inhibition assays established that OsCUL3 is necessary for
183 the proteasome-dependent degradation of OsNPR1, indicating that OsNPR1 is also a
184 substrate of CRL3 (Liu *et al.*, 2017). Rice CRL3 has the potential to influence a large
185 transcriptional immune programme, as accumulation of OsNPR1 protein in *oscul3a* mutants
186 was associated with activation of *PR* genes. This is in agreement with previous reports
187 demonstrating that overexpression of OsNPR1 resulted in constitutive activation of immune
188 genes and resistance to bacterial blight (Chern *et al.*, 2001; Chern *et al.*, 2005; Yuan *et al.*,
189 2007). However, genome-wide transcript profiling of *OsNPR1* knock down lines showed that
190 its function as an activator of immune genes was relatively modest in comparison to its role
191 in transcriptional suppression. OsNPR1 directly or indirectly down regulated the expression
192 of genes involved in photosynthesis and in chloroplast translation and transcription,
193 suggesting it plays an important role in resource reallocation during establishment of
194 immunity (Sugano *et al.*, 2010). How CRL3-mediated ubiquitination of OsNPR1 affects
195 suppression of these growth and development related genes currently remains unknown.
196 Considering CRL3 controls the cellular levels of OsNPR1, it is expected that CRL3 plays an
197 important role in limiting the suppressive effects of NPR1, thereby managing the balance
198 between defence and growth (Figure 1).
199

200 **Composition and potential structure of CRL3 ligases in SA signalling**

201 To recruit substrates CRL3 ligases utilize specific substrate adaptors that typically contain a
202 BTB domain and one or more additional protein-protein interaction domains. The BTB
203 domain and adjacent sequences directly interact with CUL3 (Canning *et al.*, 2013; Stogios *et*
204 *al.*, 2005; Zhuang *et al.*, 2009), 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 (Canet *et al.*, 2012; Hepworth *et al.*, 2005), NPR1–4 have all been implicated in SA-dependent immune signalling (Canet *et al.*, 2010; Cao *et al.*, 1997; Fu *et al.*, 2012; Liu *et al.*, 2005; Zhang *et al.*, 2006). 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 these proteins recruit NPR1 for ubiquitination by a $\mathrm{CRL3}^{\mathrm{NPR3/4}}$ 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 crystallisation 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 dimerisation has been suggested to improve the efficiency by 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 adapters, it has been demonstrated for two hetero-oligomerising F-box proteins, Pop1p and Pop2p, which are part of an Skip-Cullin-F-box (SCF, also known as CRL1) ligase in fission yeast (Seibert *et al.*, 2002). While heterodimeric $\mathrm{SCF}^{\mathrm{Pop1p\text{-}Pop2p}}$ target the cyclin-dependent kinase inhibitor Rum1p for degradation, homodimeric $\mathrm{SCF}^{\mathrm{Pop1p}}$ and $\mathrm{SCF}^{\mathrm{Pop2p}}$ complexes probably have different substrate preferences. Thus heterodimer or even heterooligomer formation between different NPR proteins potentially increases combinatorial diversity in substrate preference that could extend well beyond NPR1 as the sole substrate.

**Coactivator 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 (Figure 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 (Figure 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, 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 utilise 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 hormone

279 perception may be common a mechanism of perception by CRL ubiquitin ligases. Distinct
280 from other hormones, however, SA also regulates how and when NPR3 and NPR4 interact
281 with NPR1. This is a consequence of the vastly different affinities NPR3 and NPR4 have for
282 SA. Whereas NPR3 has a relatively low affinity for SA, NPR4 displays much higher affinity.
283 Moreover, SA binding has opposing effects on substrate recruitment. The result is that
284 elevated levels of SA disrupt NPR1-NPR4 interactions, but promote NPR1-NPR3
285 interactions (Fu *et al.*, 2012). Thus, in absence of pathogen threat when low levels of SA are
286 present, the $CUL3^{NPR4}$ ubiquitin ligase complex is thought to suppress NPR1 monomer
287 levels to prevent autoimmunity. Conversely, when cellular levels of SA increase following
288 pathogen attack, NPR1 recruitment is switched from $CUL3^{NPR4}$ to $CUL3^{NPR3}$, which is
289 necessary for local and systemic immune responses (Fu *et al.*, 2012; Furniss and Spoel,
290 2015). Although details of the mechanisms by which SA regulates $CRL3^{NPR3/NPR4}$ remain
291 largely unknown, the clear analogy to other CRL-dependent signalling pathways
292 demonstrates that SA is a key small molecule involved directly in the regulation of NPR1
293 stability and therefore likely also NPR1 transcriptional potency.
294 Endogenous signals originating from hormone signalling pathways may also
295 modulate CRL3-mediated ubiquitination and degradation of NPR1 coactivator. The
296 developmental hormone abscisic acid (ABA) interacts antagonistically with SA-dependent
297 immune signalling. Some pathogens hijack this antagonism by inducing apparent increases
298 in ABA biosynthesis, thereby inhibiting signalling steps both up and downstream of SA
299 accumulation (de Torres-Zabala *et al.*, 2007; Mohr and Cahill, 2007; Yasuda *et al.*, 2008). A
300 recent report investigated how ABA might impact SA signalling by examining NPR1 stability
301 (Ding *et al.*, 2016). It was found that treatment with ABA strongly reduced the cellular level of
302 NPR1 in a $CRL3^{NPR3/NPR4}$ and proteasome-dependent manner, suggesting that in
303 unchallenged cells ABA antagonised SA signalling by destabilising NPR1. However, by
304 changing the timing of pharmacological applications of SA and ABA, it was found that ABA
305 reduces NPR1 protein levels only if ABA treatment preceded SA treatment. These data
306 suggest that ABA has less control over the stability of SA-induced NPR1. Indeed,
307 phosphorylation of Ser11/15, which is necessary for SA-induced NPR1 degradation,
308 appeared to block ABA-induced NPR1 instability. Nonetheless, ABA treatment could strongly
309 reduce SA-induced *PR-1* gene expression, implying that antagonisms between SA and ABA
310 is more complex than can be observed by examining NPR1 protein levels at a single time
311 point. By temporally surveying NPR1 protein levels during infection by the virulent bacterial
312 pathogen *Pseudomonas syringae*, the authors revealed that SA and ABA accumulate
313 sequentially, which may allow a switch from SA-induced NPR1 protein degradation to ABA-
314 induced turnover (Ding *et al.*, 2016). The functional outcome of switching between
315 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 coactivators. 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 coordination between ABA- and SA-induced phosphorylation events orchestrate diverse pathways of NPR1 ubiquitination and degradation, each with distinct transcriptional outputs.

In addition to endogenous inputs, exogenous signals may also modulate transcriptional coactivator 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 it directly targets the UPS (Üstün *et al.*, 2016). Other pathogen effectors have also been identified to directly target the proteasome. 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 Bornke, 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 Bornke, 2015). These findings suggest that only ATP-bound RPT6 is recognised 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 coactivator, likely preventing full-scale activation of SA-responsive immune genes (Spoel *et al.*, 2009; Üstün and Bornke, 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 supress its

353 transcriptional coactivator activity. Indeed, recent work identified the *P. syringae* effector
354 avrPtoB, a U-box type E3 ligase, as an interactor of NPR1 (Chen *et al.*, 2017). Curiously, SA
355 enhanced interaction between avrPtoB and NPR1, leading to NPR1 ubiquitination and
356 subsequent proteasomal degradation. Unlike immune-induced NPR1 ubiquitination and
357 turnover, avrPtoB-induced degradation of NPR1 negatively affected SA-responsive gene
358 expression and immunity (Chen *et al.*, 2017). This suggests that avrPtoB either ubiquitinates
359 distinct Lys residues in NPR1 as compared to CRL3 or it targets NPR1 for ubiquitination
360 prior to its recruitment to CRL3. As SA promotes interaction between avrPtoB and NPR1, it
361 is plausible that avrPtoB titrates NPR1 away from SA-mediated binding to the CRL3
362 substrate adaptor NPR3.
363
364 **Degradation of other SA-responsive transcriptional immune regulators**
365 The UPS may control SA-responsive gene expression in ways that go well beyond
366 regulating the stability of master (co)activators such as NPR1 and OsWRKY45. SA-
367 responsive gene expression is modulated by many other transcriptional activators and
368 repressors from the TGA, WRKY and NIMIN or OsNRR families, many of which physically
369 interact with NPR1 and the CRL3 substrate adaptors NPR3 and NPR4 (Chern *et al.*, 2014;
370 Liu *et al.*, 2005; Saleh *et al.*, 2015; Shi *et al.*, 2013; Weigel *et al.*, 2001; Zhang *et al.*, 2006).
371 Interestingly, some of these transcriptional regulators or their close relatives have been
372 reported to exhibit UPS-dependent instability. TGA1 and TGA3 transcriptional activators
373 have been shown to interact with NPR proteins and their protein levels are regulated by
374 post-transcriptional mechanisms. Although their protein levels appeared to be controlled
375 developmentally, inhibition of the proteasome resulted in accumulation of TGA3 in the
376 nucleus (Pontier *et al.*, 2002). This suggests that TGA3 is unstable and its degradation could
377 be managed by SA. Because TGA3 interacts with NPR3 and NPR4 (Zhang *et al.*, 2006), it is
378 plausible that TGA3 is regulated by an SA-induced $CRL3^{NPR3}$ or $CRL3^{NPR4}$. Alternatively,
379 TGA3 could be targeted for degradation indirectly through its interaction with NPR1 via
380 concurrent ubiquitination and degradation. Concurrent ubiquitination and degradation of
381 multiple physically associated substrates has already been reported for a CRL3 ligase
382 involved in light signalling (Ni *et al.*, 2014). In addition to TGA transcription factors, WRKY
383 transcription factors regulate SA-responsive gene expression both positively and negatively.
384 While (in)stability of the wider WRKY protein family has not yet been examined, some
385 WRKY proteins such as the above discussed OsWRKY45 have been found to be subject to
386 UPS-dependent degradation. In Arabidopsis WRKY53 was found to be a substrate of HECT
387 domain-containing Ubiquitin Protein Ligase 5 (UPL5) during leaf senescence (Miao and
388 Zentgraf, 2010). Notably WRKY53 is also an activator of SA-responsive immune genes
389 (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 corepressors. $SCF^{COI1}$-mediated degradation of JAZ corepressors 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 corepressors. 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 corepressors, 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 corepressors 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 physically associate with the 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 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 (Crosas *et al.*, 2006; Schmidt *et al.*, 2005), indicating 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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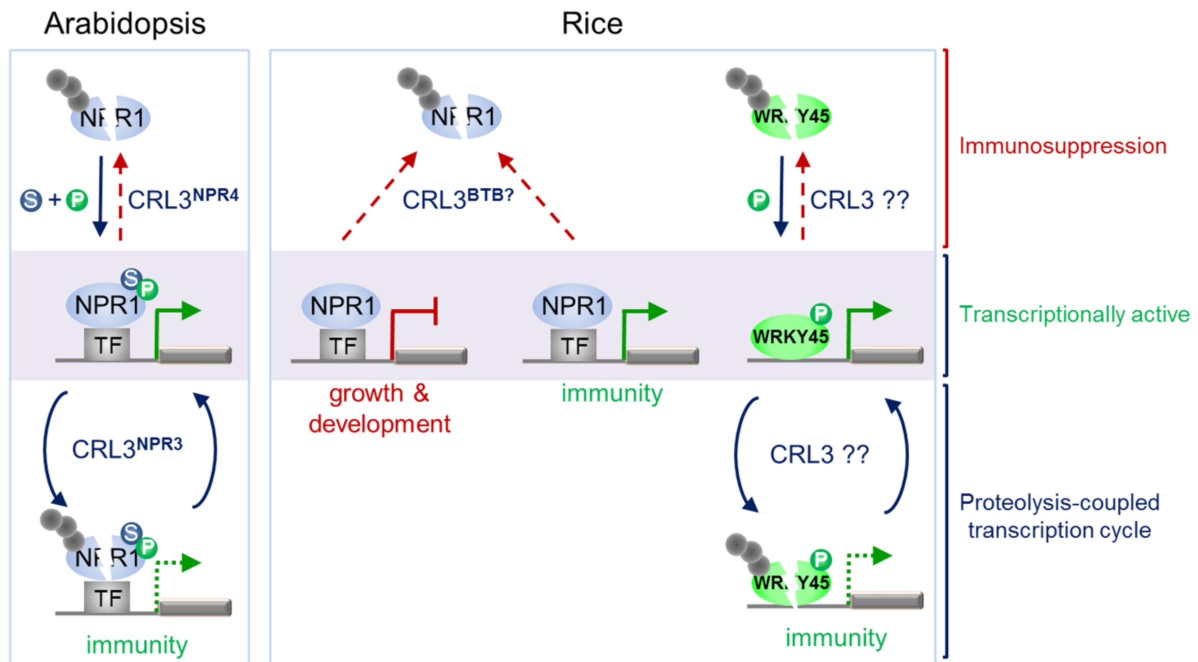


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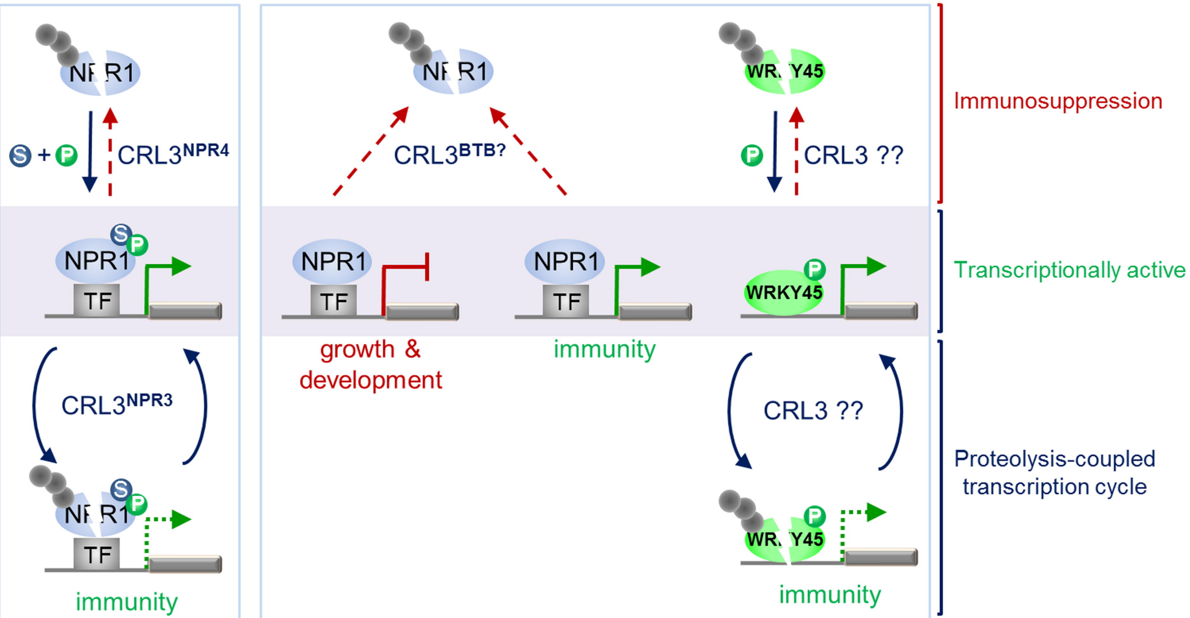
716 **Figure 1. CRL3-mediated transcriptional reprogramming in Arabidopsis and rice**
717 In Arabidopsis (left panel) stability of the transcriptional coactivator NPR1 is controlled by
718 different CRL3 ligases. In unchallenged cells NPR1 is polyubiquitinated (grey circles) by
719 $CRL3^{NPR4}$ ligases to prevent autoimmunity (*i.e.* immunosuppression). Upon pathogen
720 challenge SA induces the SUMOylation (S, blue cirlces) and phosphorylation (P, green
721 circles) of NPR1, allowing transcriptionally active NPR1 to undergo a transcription-coupled
722 proteolysis cycle that stimulates the expression of immune genes. In rice (right panel)
723 immunosuppression is accomplished by CRL3-mediated ubiquitination and degradation of
724 OsNPR1 as well as by degradation of OsWRKY45 which may also involve a CRL3 ligase.
725 Upon pathogen challenge OsNPR1 activates immune genes but also suppresses genes
726 involved in growth and development. Additionally, pathogen challenge activates
727 OsWRKY45, resulting in its phosphorylation and subsequent degradation in a proteolysis-
728 coupled transcription cycle that is hypothesised to involve a CRL3 ligase. Note that the
729 transcriptionally competent or active state of all transcription (co)regulators is represented by
730 a shaded box.


Arabidopsis
Rice
NPR1
CRL3$^{NPR4}$
CRL3$^{BTB?}$
WRKY45
CRL3 ??
Immunosuppression
TF
growth &
development
immunity
Transcriptionally active
CRL3$^{NPR3}$
Proteolysis-coupled
transcription cycle