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Plant NLRs with Integrated Domains: Unity Makes Strength

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1 Short Title: Integrated domains in plant NLRs

2

3 - one-sentence summary: Integration of unusual domains in plant immune receptors
4 is a widespread mechanism of NLR diversification enabling specific pathogen
5 detection.

6

7 - author contributions: E. G, D. T., and L. D. equally contributed in the writing of the
8 manuscript

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10

11 **Plant NLRs with integrated domains: Unity makes strength**

12

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17

18 **1. Introduction**

19 Unlike animals, plants lack an adaptive and circulating immune system. Thus, to
20 detect pathogens and generate effective defense responses, plants rely on an
21 elaborate innate immunity that involves different types of immune receptors (Cook et
22 al., 2015). Conserved pathogen-associated molecular patterns (PAMPs) are
23 recognized in the extracellular compartment of the host by cell surface-localized
24 receptors. This event triggers the activation of basal immune responses called
25 PAMP-triggered immunity (PTI). During evolution, pathogens have evolved
26 sophisticated virulence strategies to overcome host defense responses. Host-
27 adapted pathogens use an arsenal of virulence factors called effectors, that are
28 delivered into the plant cell in order to subvert diverse cellular functions (effector-
29 triggered susceptibility, ETS) through interference with PTI signaling (Jones and
30 Dangl, 2006). However, effector activities can turn against the pathogen, as they
31 often betray its presence within the cell. To recognize pathogen effectors, plants use
32 a repertoire of intracellular immune receptors that belong to a superfamily of
33 nucleotide-binding domain (NB) and leucine-rich repeat (LRR)-containing proteins
34 (NLRs). NLRs can mediate specific recognition of pathogen effectors and initiate
35 effector-triggered immunity (ETI). ETI involves transcriptional reprogramming
36 overlapping with transcriptional regulations during PTI and often provokes localized
37 host cell death at infection sites to limit pathogen spread (Jones and Dangl, 2006;
38 Maekawa et al., 2011). Adapted pathogens can evade host recognition by
39 reconfiguring their effector repertoires through various mechanisms including gain
40 and loss of effector genes, modulation of expression, and rapid evolution of effectors
41 by mutation (Arnold and Jackson, 2011; Lo Presti et al., 2015). Cycles of pathogen-
42 induced ETS and plant-mediated ETI are therefore considered as major forces
43 driving plant host-pathogen co-evolution (Jones and Dangl, 2006).

44 NLR proteins belong to STAND (Signal Transduction ATPases with Numerous
45 Domains) P-loop NTPases. Canonical plant NLRs possess a multidomain
46 architecture composed of a central nucleotide binding site (NBS) and a carboxy
47 terminal leucine-rich-repeat (LRR) domain. The NBS is believed to function, through
48 nucleotide-dependent conformational changes, as a molecular switch for NLR
49 activation (Takken et al., 2006). Depending on the nature of their N-terminal domain,
50 NLRs can be divided into two main classes: those having a Toll and interleukin-1
51 receptor (TIR) domain and those with a coiled-coil (CC) domain (CNLs) (Takken and
52 Govere, 2012). A third class, based on the presence of the N-terminal RPW8
53 domain, can also be defined. Over the last two decades, with the cloning of plant
54 NLRs and their associated effectors, molecular characterization of the mechanisms
55 employed by NLRs for specific effector recognition and signaling have been the
56 subject of intensive research.

57

58 NLR-mediated effector recognition often involves host components that bind to
59 and/or are modified by effectors (Figure 1). In the guard model, effector interference
60 with a host target (co-factor or bait) is detected by the NLR, acting as a guard of
61 modified-self (Dodds and Rathjen, 2010; Maekawa et al., 2011). Many identified
62 guarded host proteins (also referred as “guardees”) represent hubs with key immune-
63 related functions, including signaling or regulation of gene expression, and are
64 therefore commonly targeted by various effectors (Mukhtar et al., 2011; Weßling et
65 al., 2014). An effector “decoy” model has been proposed for a number of effector-
66 sensing NLRs. In this model, the guarded host protein has no defense role but
67 mimics an operational effector target and thus acts as a “decoy” that lures the
68 pathogen effector and diverts it from its real target(s) (van der Hoorn and Kamoun,
69 2008; Lewis et al., 2013; Ntoukakis et al., 2014). Studies have shown that several
70 NLRs can both detect pathogens and initiate downstream signaling, whereas other
71 NLR proteins form heterogeneous protein complexes (Césari et al., 2014; Williams et
72 al., 2014). In the core of these complexes are NLR pairs in which the two members
73 are encoded by genes arranged in a head-to-head orientation with a common
74 promoter region, which strongly suggests their co-regulation (Birker et al., 2009;
75 Saucet et al., 2015). In several cases, the two proteins of NLR pairs form a
76 heterocomplex receptor with each partner featuring specific attributes: one detects
77 pathogen effectors (the sensor) while the other functions as an inducer of disease

78 resistance (the transducer), and the signaling activity of the latter is repressed by the
79 sensor (Césari et al., 2014; Williams et al., 2014). To explain how effectors are
80 recognized by NLR pairs, an extension of the decoy model has been proposed with
81 the “integrated decoy” hypothesis (Cesari et al., 2014). Indeed, sensor NLR partners
82 were shown to contain, in addition to their conserved multidomain NLR architecture,
83 unconventional domains that are able to physically interact with their corresponding
84 effectors (Kanzaki et al., 2012; Cesari et al., 2013). Recent studies demonstrated that
85 several of these integrated domains act as decoys of effector targets, enabling the
86 sensor NLR to specifically detect pathogens (Le Roux et al., 2015; Maqbool et al.,
87 2015; Ortiz et al., 2017) (Figure 1). Perturbations of the sensor NLR are perceived by
88 the signaling partner, which activates immune signaling (Le Roux et al., 2015; Sarris
89 et al., 2015; Ortiz et al., 2017). Comparative analyses of plant immune receptor
90 architectures suggest that the integration of unusual domains (hereafter designated
91 as “IDs” for integrated domains), which potentially serve as “baits” for pathogen
92 effectors, is not restricted to paired NLRs and represent a widespread mechanism
93 (Kroj et al., 2016; Sarris et al., 2016; Bailey et al., 2018). The identification of NLR-
94 IDs signifies a breakthrough in plant NLR biology pathology, since it has profoundly
95 changed our view of how plant NLRs can function and evolve.

96 In this review, we summarize current knowledge of NLR-IDs with detailed examples,
97 discuss their genetic and functional diversity, and illustrate how the study of NLR
98 function and mode of action has led to advances in plant disease control.

100 **2. NLRs with integrated decoys: an ingenious pathogen detection mechanism**

101 Recent independent studies have provided convincing evidence that IDs enable
102 specific detection of pathogens by acting as molecular decoys that structurally mimic
103 pathogen true virulence targets to monitor host immunosuppression attempts. How
104 these integrated domains confer effector recognition and trigger activation of immune
105 signaling are very intriguing questions. Well-characterized examples of NLR-ID
106 fusions in paired NLRs include the *Arabidopsis thaliana* RRS1, which carries a
107 WRKY domain (Le Roux et al., 2015), and the RGA5 and Pik-1 proteins from rice
108 (*Oryza sativa*), both of which integrate an HMA (RATX1) domain (Ortiz et al., 2017).
109 These examples are described in detail below.

111 **The WRKY domain of RRS1-R**

112 Experimental validation of the integrated decoy model was first provided for the
113 *Arabidopsis/Ralstonia solanacearum* model. In 2001, Deslandes and colleagues
114 cloned a resistance gene encoding RRS1-R (RESISTANCE TO RALSTONIA
115 SOLANACEARUM1) conferring broad-spectrum resistance to the soil-borne bacteria
116 *R. solanacearum*, the causal agent of bacterial wilt (Deslandes et al., 1998;
117 Deslandes et al., 2002). RRS1-R contains at its carboxyl terminus a 'WRKY' DNA-
118 binding domain. This domain is conserved in defensive plant WRKY transcription
119 factors that orchestrate biotic stress responses through the recognition of W-box *cis*-
120 regulatory elements in gene promoters (Rushton et al., 2010). As being the first
121 cloned NLR with an extra domain, RRS1-R was initially considered an anomaly in the
122 field. Later, RRS1-R was shown to cooperate genetically and molecularly with a
123 second TNL, namely RPS4 (RESISTANCE TO PSEUDOONAS SYRINGAE4) (Birker
124 et al., 2009; Narusaka et al., 2009), to recognize effectors from different pathogens.
125 These effectors included *R. solanacearum* PopP2, a member of the YopJ superfamily
126 of acetyltransferase (Deslandes et al., 2003; Tasset et al., 2010); and AvrRps4, an
127 unrelated effector from leaf-infecting *Pseudomonas syringae* pathovar *pisi* (Hinsch
128 and Staskawicz, 1996; Sohn et al., 2012). Encoded by two co-regulated genes
129 present in a head-to-head orientation, RRS1-R and RPS4 TNLs form a functional
130 receptor recognition/signaling complex through homo- and heterodimerization
131 involving their respective TIR domains (Williams et al., 2014). Two recent studies
132 revealed that the RRS1-R/RPS4 NLR complex is activated through the targeting of
133 the RRS1-R WRKY domain by PopP2 and AvrRps4 effectors (Le Roux et al., 2015;
134 Sarris et al., 2015). Catalytically active PopP2 acetylates a key lysine residue
135 (K1221) in the invariant heptad of the WRKY domain of RRS1-R, blocking its binding
136 to W-box DNA sequences. Homology modeling predicts that K1221 acetylation
137 disrupts WRKY domain electrostatic potential at the interface with DNA. In the
138 absence of RRS1-R/RPS4 recognition, PopP2 uses this acetylation strategy to inhibit
139 WRKY-DNA binding activities and transactivation functions needed for defence gene
140 expression and basal resistance. RRS1-R WRKY domain represents therefore a
141 decoy which betrays the defense-suppressing abilities of PopP2 and AvrRps4 on
142 their host virulence targets: the defensive WRKY transcription factors. The direct
143 fusion of a WRKY decoy domain within the RRS1-R/RPS4 NLR complex creates an
144 efficient monitoring system for the indispensable virulence activities of different
145 pathogens. Recently, Ma and colleagues demonstrated that, prior to effector

146 detection, the WRKY domain negatively regulates the RPS4-RRS1 complex through
147 specific interactions with an adjacent domain in RRS1 (Ma et al., 2018). Binding of
148 AvrRps4 to the WRKY domain disrupts these intramolecular interactions leading to
149 the derepression of RRS1. Therefore, besides its effector sensing function, the
150 integrated WRKY domain of RRS1 also has an important regulatory role in
151 preventing inappropriate receptor activation in the absence of pathogens.

152

153 **The HMA domain of RGA5 and Pik-1**

154 The study of the RGA4/RGA5 receptor NLR pair in rice has enabled significant
155 progress in deciphering the mode of action of paired NLRs. This NLR pair cooperates
156 genetically and physically in the recognition of AVR-PiA and AVR1-CO39, two
157 unrelated effectors of the rice blast fungus *Magnaporthe oryzae* (Okuyama et al.,
158 2011; Cesari et al., 2013). In the absence of the pathogen, constitutive disease
159 resistance and cell death mediated by RGA4 is repressed by RGA5 through the
160 formation of heteroprotein complexes. The C-terminal part of RGA5 contains a Heavy
161 Metal-Associated (HMA) domain, initially found in a cytoplasmic copper chaperone in
162 *Saccharomyces cerevisiae*, which can directly interact with AVR-PiA and AVR1-
163 CO39, enabling pathogen recognition. Physical association of the AVR-PiA effector
164 with the HMA domain of RGA5 triggers cell death through RGA4 de-repression
165 (Césari et al., 2014). Interestingly, recognition of the AVR-Pik effector of *M. oryzae* by
166 the unrelated CC-NLR pair Pik-1/Pik-2 in rice is also triggered by direct binding to an
167 HMA domain in Pik-1 that, contrary to RGA5, is integrated between its CC and NB
168 regions (Ashikawa et al., 2008; Maqbool et al., 2015). The different location of the
169 HMA domain in RGA5 and Pik-1 suggests that these domains have been fused to
170 those two unrelated NLRs through independent events (Cesari et al., 2013). Although
171 HMA domain-containing proteins have not been previously described as effector
172 targets, the presence of a HMA domain in the rice Pi21 factor, which is required for
173 full susceptibility to the rice blast fungus (Fukuoka et al., 2009; Zhang et al., 2016),
174 supports the idea that the HMA domains of RGA5 and Pik-1 are decoys for AVR-PiA,
175 AVR1-CO39, AVR-Pik, and functionally related effectors. Determination of the crystal
176 structure of AVR-PikD complexed with a dimer of the Pikp-1 HMA domain revealed
177 that key residues at the interaction interface are required for effector binding and
178 recognition (Maqbool et al., 2015). In addition, variations at binding interfaces
179 between AVR-Pik effector variants and HMA domains of Pik alleles were found to

180 determine recognition specificity. Such recent findings highlight how new receptor
181 specificities arise from natural selection (De la Concepcion et al., 2018). How the
182 binding of effectors to HMA domains can trigger activation of immune signaling
183 remains unknown. It is hypothesized that effector binding promotes NLR domain
184 rearrangements leading to immune complex activation (Césari et al., 2014). Binding
185 of AVR-PiA to RGA5 HMA domain is also necessary for pathogen recognition, but
186 protein-protein interaction analyses revealed that moderate affinity to mutated AVR-
187 PiA proteins still confers recognition (Ortiz et al., 2017). Furthermore, additional sites
188 in RGA5, outside the ID, are suspected to mediate interaction with the effector. Thus,
189 the juxtaposition of integrated decoy domains with additional NLR sites interacting
190 properties creates a highly resilient surveillance system. How the binding of effectors
191 to HMA domains can trigger immune signaling remains unknown. It is hypothesized
192 that effector binding promotes NLR domain rearrangements leading to immune
193 complex activation (Césari et al., 2014).

194

195 **The NOI/RIN4 domain of Pii-2**

196 Pathogen detection by paired NLR-IDs is not restricted to the direct recognition
197 model. Indeed, IDs might also function in indirect recognition by perceiving
198 modifications of a host protein targeted by an effector. This concept is supported by a
199 study of the unconventional NOI/RIN4 domain of the rice NLR-ID Pii-2 that is
200 hypothesized to monitor, through direct binding, the integrity of the OsExo70-F3 host
201 protein, a target of the *M. oryzae* effector AVR-Pii (Fujisaki et al., 2017).

202

203 **3. NLR-IDs : a mechanism of NLR diversification**

204 The search for protein domains associated with typical NLR domains in public
205 databases made it possible to identify entire NLR-ID directories and to analyze their
206 structure in many plants. Already present in mosses, NLR-IDs occasionally represent
207 a large proportion of NLRs in terrestrial plants (Kroj et al., 2016; Sarris et al., 2016;
208 Bailey et al., 2018; Stein et al., 2018) (Table1). Kroj et al. (2016) detected 162
209 hypothetical IDs across 33 genomes by looking for interpro domains using the
210 GreenPhyl database. Although their analysis was not exhaustive due to potential
211 misannotations of the applied databases, they identified a high diversity of IDs (90
212 different domains). Sarris et al. (2016) reported 265 unique ID fused to NLRs in 37
213 genomes of land plants. More recently, Bailey et al. (2018) identified NLR-IDs in 9

214 grass species. 31 types of different domains were mainly represented in these
215 species. By focusing on 13 *Oryza* species, Stein et al. (2018) described a highly
216 variable structure of genes coding for several hundreds of different NLR-ID proteins.
217 They were able to detect a significant enrichment for these NLR-IDs within pairs of
218 genes arranged in a head-to-head configuration in the genomes. The widespread
219 distribution of NLR-IDs, despite their low abundance in some plant genomes (see
220 Table 1), suggests a successful evolutionary mechanism of NLR diversification
221 commonly used by plants to expand their pathogen recognition capabilities, allowing
222 them to cope with highly and rapidly adaptable pathogen-derived molecules.
223 Accordingly, the IDs identified in these studies, are derived from proteins which are
224 extremely diverse in sequence and predicted molecular functions. The most frequent
225 domains found in NLR-IDS include the WKRY and BED zinc finger (Znf-BED) DNA-
226 binding domains, and the protein kinase domains. The decoy function of the WRKY
227 domain in the RRS1-R NLR has been already validated (see above) (Le Roux et al.,
228 2015; Sarris et al., 2015). The BED-zinc finger domain was originally identified in
229 transposases and transcription factors (Hayward et al., 2013) but, contrary to RRS1-
230 R, the targeting by pathogen effectors remains to be demonstrated.
231 However, Kroj and colleagues showed that the ZBED NLR protein from rice,
232 containing three BED domains, is required for resistance to *M. oryzae* (Kroj et al.,
233 2016). In response to the pathogen, ZBED over-expressing lines were more
234 resistant, whereas a *zbed* null mutant showed increased susceptibility. These data
235 strongly suggest that the BED domains in ZBED NLR proteins represents decoys that
236 mimic host BED proteins targeted by *M. oryzae* effectors. The Xa1 NLR from rice,
237 which confers resistance against isolates of the bacterial blight pathogen
238 *Xanthomonas oryzae* by recognizing multiple TALEs (Yoshimura et al., 1998; Ji et al.,
239 2016), contains a Znf-BED domain in its N-terminal part. The mechanism that allows
240 Xa1 to recognize TALEs remains to be elucidated. It is tempting to speculate that
241 Xa1 Znf-BED domain might also act as a decoy to lure TALEs that target host Znf-
242 BED proteins, for the subversion of host gene expression (Zuluaga et al., 2017).
243 Also the functionality of NLR IDs with predicted catalytically active protein kinase
244 domains need to be experimentally validated. However, their sensing abilities can be
245 deduced from well described examples of kinases acting as decoys that physically
246 interact with classical NLRs, e.g. the kinases Pto and PBS5 interacting with the NLRs
247 Prf and RPS5, respectively (Khan et al., 2016). It is noteworthy that in primitive land

248 plants the fusion of Kinase domains or DUF676 to NBS-LRRs that lack CC or TIR
249 domains has been described (Gao et al., 2018), suggesting that these kind of IDs
250 could ensure the signaling function of these missing domains. Therefore, such IDs in
251 NLR proteins could fulfill either sensor or signaling functions, or both.

252 Interestingly, there are significant overlaps between IDs and protein domains
253 previously identified as interacting partners of effectors in interactome screens
254 (Mukhtar et al., 2011; Weßling et al., 2014; Sarris et al., 2016), including well-
255 characterized guardees or decoys. Examples are the exocyst complex factor Exo70,
256 required for recognition of AvrPii by NLR Pii in rice (Fujisaki et al., 2015), and RIN4, a
257 target of multiple effectors that is guarded by RPS2 and RPM1 NLRs in Arabidopsis
258 (Mackey et al., 2002; Mackey et al., 2003; Kim et al., 2005). Such overlaps strongly
259 suggest that IDs could act as sensor/decoy by mimicking effector targets. Since
260 many IDs correspond to protein domains with unknown biological activity, they
261 represent promising candidates to uncover host components targeted by effectors
262 and whose participation in various layers of plant immunity has not been assigned
263 yet.

264 Whether all the putative IDs identified in the whole genome analyses also serve as
265 sensor/decoy in immune response remains to be demonstrated. Moreover, detailed
266 investigations on gene structure and function should help to reduce false positive
267 among computationally predicted NLR-IDs (Giannakopoulou et al., 2016), and shed
268 light on new resistance mechanisms.

269

270 **4. Mechanisms involved in NLR-ID fusion events**

271 Within NLR-IDs, the majority of IDs appear as singular N- or C-terminal domains.
272 However, in some cases the fusion of several domains in the same protein is
273 observed. For example, AtWRKY19 NLR in Arabidopsis integrates both an N-
274 terminal WRKY domain and a C-terminal kinase domain. In a minority of cases,
275 including rice Pik-1, integration has occurred between the N-terminal signaling
276 domain and the central nucleotide binding domain of the NLR. These observations
277 indicate that some NLRs can tolerate integration of sensor domains at various
278 positions in their modular architecture, while maintaining their signaling functions.
279 Bailey *et al.* recently investigated the evolutionary dynamic of NLR-IDs in the
280 genomes of nine grass species. They concluded that NLR-IDs in grasses were not
281 evenly distributed across their phylogeny, but a specific clade with up to 58% of

282 NLRs containing IDs was observed (Bailey et al., 2018). In this clade, they
283 highlighted an amino acid sequence motif located immediately upstream of the fusion
284 site, which could play an important role in the integration process. They proposed
285 that DNA transposition and/or ectopic recombination is a major driving force behind
286 domain integration in grasses; repeated independent integration events were
287 observed, suggesting that integration occurred frequently and independently during
288 evolution, giving rise to a high diversity of IDs. Similarly, Brabham *et al.* recently
289 showed that orthologs of the RGH2 NLR from species across the grasses were
290 subject to large variation in domain structure, including the presence/absence of an
291 integrated Exo70 domain (Brabham et al., 2018). These trans-species
292 polymorphisms provided an opportunity to follow the molecular evolution of the
293 *Exo70* gene families and to investigate the role of Exo70 as an integrated domain in
294 the RGH2 NLR. This study showed that upon pathogen pressure, non-integrated
295 *Exo70* genes are under strong purifying selection, whereas they are under relaxed
296 purifying selection when integrated into RGH2. Across the *Oryza* genomes, the
297 presence of IDs in 17 different NLR subfamilies (from a total of 36) point to multiple
298 and independent acquisition of IDs (Stein et al., 2018).

299

300 **5. NLR-IDs: towards the elucidation of additional NLRs functions?**

301 Besides the well-documented role of NLRs for innate immunity in both animals and
302 plants, additional functions controlled by NLRs are currently discussed. In animals,
303 NLRs play a role in developmental processes, such as spermatogenesis and fertility,
304 suggesting a control of the reproductive system by NLR proteins (Meunier and Broz,
305 2017). In plants, inappropriate activation of NLRs caused by mutations or
306 incompatible combinations also impact development (Chae et al., 2014; Chen et al.,
307 2016; Atanasov et al., 2018; Chakraborty et al., 2018). The integrated decoy model
308 predicts an important role of IDs in pathogen detection. Beyond their function of
309 effector sensor, some IDs might have retained the biological activities of the proteins
310 from which they are derived. Thus, additional functions controlled by NLRs in plants
311 could be revealed by looking at IDs.

312 In this regard, the integration of a BED domain, one of the most frequently found ID in
313 plant species, gained attention. This domain appears to be shared by transposases
314 and by proteins that perform critical cellular functions (Aravind, 2000; Hayward et al.,
315 2013). The BED-zinc finger domain of the Tam3 (Transposase of Activator from

316 maize) transposase has been shown to suppress the DNA TAM3 transposon activity
317 in *Anthirrhinum majus* by re-localizing the Tam3 transposase out of the nucleus
318 (Zhou et al., 2017). More globally, BED-related IDs could be sensors of cellular
319 homeostasis perturbation by environmental stress which rely in part on DNA
320 transposition control (Negi et al., 2016).

321 In the case of RRS1, inhibition of its DNA binding activity provoked by particular
322 mutations in its WRKY domain leads to autoimmunity in a RPS4-dependent manner
323 (Noutoshi et al., 2005; Sohn et al., 2014). Therefore, RRS1-R was initially considered
324 a negative regulator of immune-related genes. The autoimmune phenotype of the
325 RRS1-R *slh1* variant is conditioned by low humidity, suggesting that RRS1-R,
326 besides its function in pathogen recognition, could also sense particular
327 environmental disturbances such as drought stress. In response to specific biotic and
328 abiotic stresses, RRS1-R likely acts directly at genomic DNA and, together with
329 RPS4, behave as a reactive switch for transcriptional and signaling reprogramming.
330 Whether NLR-IDs possess broader sensing functions remains to be determined, but
331 almost certainly the functional characterization of their IDs will help to elucidate
332 potential additional NLRs functions.

333

334 **6. Towards the engineering of synthetic NLR-IDs with extended recognition** 335 **capabilities**

336 The high diversity of NLRs provides plants with versatile options for effective plant
337 immunity. However, on one hand, the ability of effectors to evolve rapidly and, on the
338 other hand, the necessary fine-tuning of NLR functions to avoid autoimmunity (see
339 Box 1) restrict the possibilities to transfer immune receptors into other plant genomes
340 for improved crop protection. Hence, strategies for NLR engineering are of particular
341 interest in plant immunity research. If successful, it could be possible to modify NLR
342 recognition specificity, or alternatively widen the range of effector recognition while
343 making it less feasible for pathogens to bypass NLRs. One possibility is to alter the
344 structure of NLR proteins itself. For example, a study conducted by Segretin et al.
345 showed that few amino acid changes in the LRR domain of R3a in potato (*Solanum*
346 *tuberosum*) enabled this NLR to recognize another isoform of AVR3a from
347 *Phytophthora infestans* (Segretin et al., 2014). However, direct NLR effector
348 recognition (modified self) is likely to be less tolerant to variations compared to the
349 indirect and integrated decoy models. Therefore, decoy engineering represents a

350 more suitable approach. A proof-of-concept study was recently performed by Kim
351 and colleagues (2016), demonstrating successfully the modification of the
352 Arabidopsis protein kinase PBS1 that represents a decoy for pathogen-derived
353 proteases (Kim et al., 2016). PBS1 is involved in basal immune response and acts as
354 target/decoy for the effector AvrPphB from *Pseudomonas syringae*, which can cleave
355 PBS1 due to its protease activity (Shao et al., 2003; Ade et al., 2007). PBS1
356 cleavage products are recognized by the NLR protein RPS5 (DeYoung et al., 2012)
357 that in turn initiates resistance signaling. For a modified PBS1 decoy variation, the
358 proteolytic target site, which is normally cleaved by AvrPphB, is exchanged with
359 another proteolytic site. This modification enables the cleavage of PBS1 by other
360 effectors, such as AvrRpt2 of *P. syringae*, Nla protease of Tobacco Etch Virus (TEV)
361 and Nla protease of Turnip Mosaic Virus (TuMV). AvrRpt2 also triggers activation of
362 RPS2 NLR by cleaving a nitrate-induced (NOI) domain present in RIN4, a negative
363 regulator of plant defense which is guarded by multiple NLR proteins (Mackey et al.,
364 2002; Mackey et al., 2003; Kim et al., 2005). Such protease cleavage sites are found
365 in IDs present in a subset of NLRs, paving the way to the design of single NLR-IDs
366 combining multiple protease recognition sequences.

367 The modifications of NLR IDs, as well as the replacement of IDs with other identified
368 effector targets from the host genome, display other promising tools to create novel
369 effector traps. Considering that modifications of NLRs as well as of their IDs can
370 compromise homo/heterocomplex formation required for their function and trigger
371 either inactivation or autoactivation of the receptor, it is important for this approach to
372 identify which IDs and which NLRs are best suited for fusion manipulation (e.g. IDs
373 swapping/shuffling). For this, we need to gain better knowledge of interaction sites
374 within NLR and its IDs, but also of the interaction of sensor and signaling NLRs.
375 Furthermore, it is important to determine the layout of effector-ID complexes.
376 Recently, the structure of several complexes has been resolved (Maqbool et al.,
377 2015; Ortiz et al., 2017; Zhang et al., 2017). Determination of the molecular and
378 structural bases of these interactions is crucial for the successful design of synthetic
379 “multi-sensor NLR-IDs” made from the juxtaposition of different IDs, giving them
380 extended recognition capabilities.

381

382 **Conclusion**

383 The past 20 years have brought major advances in plant NLR biology. The discovery
384 of NLR-IDs represents a significant step towards a better understanding of
385 mechanisms involved in the evolution and function of NLRs. During plant evolution,
386 NLR-IDs appeared independently several times and in different configurations.
387 Besides the sensing and regulating functions of IDs, important questions remain
388 unanswered (see “Outstanding questions” box). There is still a critical need for further
389 in-depth studies to establish the biological functions of IDs fused to NLRs and to
390 elucidate the molecular events which link NLR-IDs activation with immunity
391 pathways. Overall, NLR-IDs provide promising tools for the design of new strategies
392 to protect plants against pathogens. Nevertheless, it remains to be determined
393 whether engineered synthetic NLR-IDs can provide sustainable disease resistance,
394 especially in different crop species.

395

396

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400 more extensively because of space limitations.

401

402

403

404 Figure legends.

405

406 Figure 1:

407 NLRs can directly or indirectly detect the presence of pathogen effectors by
408 monitoring the manipulation of their host targets (baits or decoys, a). According to the
409 integrated decoy model, integrated domains (IDs) in NLRs behave as decoy of
410 effector targets enabling recognition of effector activities. This recognition can be
411 direct or indirect (b and c, respectively). Different studies reported the existence of
412 diverse IDs (in sequence and predicted molecular functions), which can be present at
413 various positions within the modular structure of NLRs (d). NLR-IDs can be
414 engineered using different strategies aimed at providing (i) extended specificity (i.e.,
415 specific point mutations in IDs enabling recognition of various allelic forms of a
416 pathogen effector), (ii) multi-recognition capabilities (by integrating IDs from different
417 NLRs within a single NLR), or (iii) new recognition specificities (by integrating
418 previously characterized effector targets that then act as sensors) (Nt : N-terminal
419 domain ; NBS : nucleotide binding site ; LRR : leucine-rich repeat ; E : Effector ; T :
420 effector target ; ID : integrated domain ; ET : previously characterized effector target)

421

422

423 Table 1. Overview of NLR-ID repertoires in plant species

Reference	No. of species investigated	No. of species with NLR-ID	No. of NLR-IDs	No. of NLR-IDs / species	Average percent of NLR-IDs (of all NLRs)
Kroj <i>et al.</i> , 2016	33	23	34	from 1 to 16	3.5 %
Sarris <i>et al.</i> , 2016	36	35	717	from 1 to 93	6.8 %
Bailey <i>et al.</i> , 2018	9	9	331	from 7 to 133	7.9 %
Funk <i>et al.</i> , 2018	1	1	24	24	14.0 %
Stein <i>et al.</i> , 2018	13	13	446	—	8.2 %

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629

ADVANCES

- Unusual domains integrated in the architecture of some plant NLRs (NLR-IDs) behave as decoys that mimic virulence targets of effector proteins, thus enabling pathogen detection.
- IDs in NLRs are extremely diverse in sequence and predicted molecular functions.
- The widespread distribution of NLR-IDs suggests an evolutionary mechanism of NLR diversification used by plants to expand their pathogen recognition capabilities.
- Identification of NLR-IDs paves the way to the development of novel strategies of plant disease control.

OUTSTANDING QUESTIONS

- What are the various mechanisms and components involved in the activation of NLR-ID proteins? Can we identify novel immunity components by characterizing IDs?
- Are NLR-IDs further involved in the downstream signaling mechanisms after activation by pathogen effectors?
- What additional functions controlled by plant NLRs can be revealed by analyzing NLR-IDs?
- What are the dynamics of host-pathogen co-evolution? How quickly can NLR-IDs be bypassed by pathogen effectors? What are the requirements necessary to successfully engineer NLR-IDs? What are the precautions to be considered when selecting the ID and structure modifications to ensure that novel NLR-IDs confer durable resistance, with minimal risk of functionality-loss or autoactivation?
- Is it possible to engineer a universal NLR-ID that confers broad pathogen resistance in various plant species?

BOX 1. Immunity Under Strict Control

The plant immune response is tightly adjusted to biotic stress and not activated in a non-challenging environment. In case of inappropriate defense activation, plant fitness is affected, and drastic developmental phenotypes such as tissue necrosis or dwarfism can be observed (Chakraborty et al., 2018). These phenotypes are similar to what is observed in plant hybrids when genetic incompatibility, resulting from incompatible allelic forms of parental genes, occurs (Chen et al., 2016). Chae et al. (2014) revealed that NLR alleles are responsible for most incompatibilities in *A. thaliana* (Chae et al., 2014). Accordingly, mutations in NLRs efficiently suppress hybrid incompatibility (Atanasov et al., 2018). NLR-IDs that negatively regulate the function of signaling NLRs could play a major role for appropriate activation of immune-related mechanisms. Hence, mutations in IDs can be responsible for autoactivation of NLRs. This is exemplified by the *slh1* (susceptible to low humidity1) mutation in the integrated WRKY domain of RRS1 that, under drought conditions, leads to constitutive activation of plant defense and a severe autoimmune dwarf phenotype (Noutoshi et al., 2005). A similar phenotype is obtained upon expression of an RRS1-R variant

mimicking a modification of the WRKY domain by its cognate effector and triggering constitutive activation of the RPS4/RRS1-R complex (Le Roux et al., 2015).

This very sensitive recognition device is accompanied by a functional separation with signaling, through the involvement of NLR pairs: one NLR detects the presence of the pathogen, the other initiate downstream signaling (see Introduction). Independent evolution of the two NLR proteins adds flexibility to ensure the effectiveness of the immune system. New effectors can thus be recognized and activate existing signal pathways. It is also possible to modify signaling without affecting the recognition of the effectors, this can especially be useful in changing environmental parameters. A strict control of NLR structure and expression is therefore essential to ensure plant fitness and development. Hence, to avoid a dramatic reduction of plant vigor in future transgenic plants expressing engineered NLR-IDs, and to promote durable resistance engineering, it is necessary to perform more extensive structural analyses of NLR-IDs, and to gain a more comprehensive understanding of the mechanisms controlling their activation.

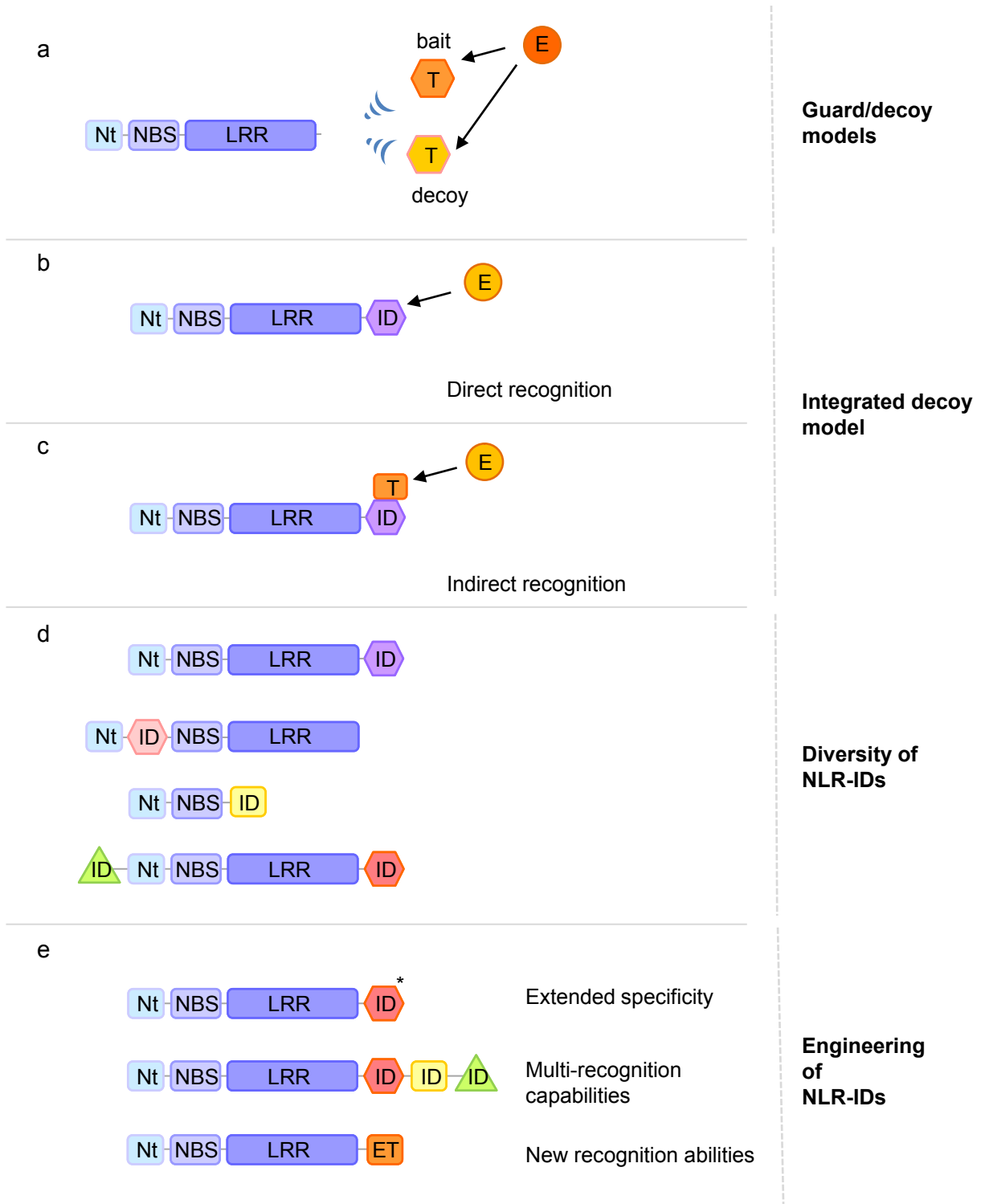


Figure 1: Different mechanisms of effector recognition mediated by NLR-IDs

NLRs can directly or indirectly detect the presence of pathogen effectors by monitoring the manipulation of their host targets (baits or decoys, a). According to the integrated decoy model, integrated domains (IDs) in NLRs behave as decoy of effector targets and enable recognition of effector activities. This recognition can be direct or indirect (b and c, respectively). Different studies reported the existence of diverse IDs (in sequence and predicted molecular functions) which can be present at various positions within the modular structure of NLRs (d). NLR-IDs can be engineered using different strategies aimed at providing (I) extended specificity (i.e. specific point mutations in IDs enabling recognition of various allelic forms of a pathogen effector), (II) multi-recognition capabilities (by integrating IDs from different NLRs within a single NLR), or (III) new recognition specificities (by integrating previously characterized effector targets that then act as sensors) (Nt : N-terminal domain ; NBS : nucleotide binding site ; LRR : leucine-rich repeat ; E : Effector ; T : effector target ; ID : integrated domain ; ET : previously characterized effector target).

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