

1 **Polymorphic residues in an allelic rice NLR expand binding and**
2 **response to effectors of the blast pathogen**

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30 **Abstract**

31 Accelerated adaptive evolution is a hallmark of plant-pathogen interactions. Plant
32 intracellular immune receptors (NLRs) often occur as allelic series with differential
33 pathogen specificities. The determinants of this specificity remain largely unknown.
34 Here, we unravelled the biophysical and structural basis of expanded specificity in the
35 allelic rice NLR receptor Pik, which responds to the effector AVR-Pik from the rice
36 blast pathogen *Magnaporthe oryzae*. Rice plants expressing the Pikm allele resist
37 infection by blast strains expressing any of three AVR-Pik effector variants, whereas
38 those expressing Pikp only respond to one. Unlike Pikp, the integrated HMA domain
39 of Pikm binds with high affinity to each of the three recognised effector variants, and
40 variation at binding interfaces between effectors and Pikp-HMA/Pikm-HMA
41 domains encodes specificity. By understanding how co-evolution has shaped the
42 response profile of an allelic NLR, we highlight how natural selection drove the
43 emergence of new receptor specificities. This work has implications for engineering of
44 NLRs with improved utility in agriculture.

45 Introduction

46 The innate immune systems of plants and animals monitor the extracellular space and
47 intracellular environment for the presence and activities of microbial pathogens^{1,2}. In
48 plants, immune receptors of the NLR (nucleotide-binding, leucine-rich repeat)
49 superfamily monitor the intracellular space for signatures of non-self, typically
50 detecting translocated pathogen effector proteins either by direct-binding, or
51 indirectly via monitoring their activity on host targets^{3,4}. Co-evolution between
52 pathogens and hosts has driven diversification of plant NLRs, with many NLR genes
53 present in allelic series, with distinct effector recognition profiles⁵⁻¹⁵. Pathogen effectors
54 can show strong signatures of positive selection including high levels of non-
55 synonymous (resulting in amino acid changes) over synonymous
56 polymorphisms^{5,7,12,16-18}. How NLR and effector diversification contributes to gene-for-
57 gene immunity in plants is poorly understood. Defining how allelic NLRs recognise
58 and respond to specific pathogen effectors offers new opportunities to engineer control
59 of plant diseases^{19,20}, leading to improved global food security.

60 Many NLRs function synergistically, with some acting as a “sensors”, to detect
61 pathogens, and others as “helpers”, required for initiation of immunity^{1,21,22}. These
62 NLRs can be genetically linked in pairs, with a shared promoter^{21,23-26}, or unlinked but
63 part of a complex genetic network²⁷. One mechanism of effector recognition by sensor
64 NLRs is via unconventional integrated domains that likely have their evolutionary
65 origin as host effector targets²⁸⁻³¹. Such integrated domains can act as “baits” to target
66 effectors by direct binding, or act as substrates of an effector’s enzymatic activity^{28,31}.
67 Genetically paired NLRs with integrated domains have repeatedly evolved in rice^{29,30},
68 and can detect effectors from the rice blast pathogen *Magnaporthe oryzae* (syn.
69 *Pyricularia oryzae*), the causative agent of the most devastating disease of rice - the
70 staple crop that feeds more than half the world population^{5,25,26,32}.

71 The rice NLR pair Pik is comprised of Pik-1 (the sensor) and Pik-2 (the helper). This
72 receptor pair responds to the *M. oryzae* effector AVR-Pik by direct binding to an
73 integrated HMA (heavy metal-associated) domain, positioned between the CC (coiled-
74 coil) and nucleotide-binding (NB) domains of Pik-1³³ (**Fig. 1a**). Both the AVR-Pik
75 effectors and the Pik NLRs exist as an allelic series in *M. oryzae* and rice respectively,
76 most likely arisen through co-evolutionary dynamics between pathogen and host^{5,34,35}.
77 As such, they represent an excellent system for understanding the mechanistic basis
78 of recognition in plant immunity. Comparison of amino acid sequence identity
79 between the domains of paired Pik NLR alleles shows the integrated HMA domain is
80 the most polymorphic region³⁵ (**Fig. 1a,c**), consistent with this being the direct binding
81 region for the AVR-Pik effectors. The HMA domain also contains variable amino acids
82 that have been used as a markers for Pik allele identification in rice³⁵. In addition, AVR-
83 Pik is a remarkable example of an effector with an extreme signature of positive
84 selection, as all known AVR-Pik nucleotide polymorphisms are non-synonymous,
85 resulting in amino acid changes^{16,18} (**Fig. 1b**). Further, these polymorphisms map to

86 interface residues identified in the crystal structure of the effector variant AVR-PikD
87 bound to the HMA domain of the NLR allele Pikp³³, suggesting they are adaptive.

88 While rice plants expressing the NLR allele Pikp are resistant to *M. oryzae* strains
89 expressing the effector variant AVR-PikD, rice plants expressing the allele Pikm
90 respond to strains expressing AVR-PikD, AVR-PikE, or AVR-PikA³⁴ (**Fig. 1b**).
91 Importantly, neither Pikp nor Pikm respond to the stealthy effector variant AVR-PikC,
92 which evades detection by any known Pik NLR³⁴. The molecular mechanism by which
93 Pik NLR variation acts to expand effector recognition remains unclear.

94 Previous work established the structural basis of AVR-PikD recognition by the Pikp-1
95 NLR³³. Here, we reveal how co-evolutionary dynamics between a pathogen and a host
96 has driven the emergence of new receptor specificities. By taking advantage of our
97 ability to reconstruct complexes between Pik-HMA domains and AVR-Pik effectors,
98 and to recapitulate cell death responses (indicative of immunity) in the model plant
99 *Nicotiana benthamiana*, we show a correlation between protein binding affinities, and
100 activation of immunity. By obtaining crystal structures of the Pikm-HMA domain in
101 complex with three different AVR-Pik variants, we define the interfaces that support
102 expanded effector recognition. We also obtained new structures of the Pikp-HMA
103 domain in complex with the recognised effector AVR-PikD, but also with the
104 unrecognised AVR-PikE. Together, these structures establish a previously
105 unappreciated role for the C-terminus of the HMA domain in mediating effector
106 interaction. Understanding how host NLRs have evolved new specificities in response
107 to pathogen effectors highlights the potential to engineer new-to-nature receptors with
108 improved functions such as recognition of stealthy effector variants, and has broad
109 implications for rational design of plant NLRs.

110 **Results**

111 **Pikm-mediated cell death in *N. benthamiana* recapitulates allele-specific effector**
112 **responses in rice**

113 Pikp-mediated cell death in *N. benthamiana* phenocopies effector variant-specific
114 resistance in rice, with Pikp responding to AVR-PikD, but not AVR-PikE, AVR-PikA,
115 or AVR-PikC³³. Here, we show that Pikm responds to each of AVR-PikD, AVR-PikE,
116 or AVR-PikA, but not to AVR-PikC, in this assay (**Fig. 1d,e, Table 1**). These results
117 match the response of rice cultivars expressing Pikm to *M. oryzae* strains encoding the
118 effectors³⁴. Interestingly, we observe a qualitative hierarchy in the level of Pikm-
119 mediated cell death in response to the effectors in the order AVR-PikD > AVR-PikE >
120 AVR-PikA (**Fig. 1d,e**). To allow for direct comparison, we repeated this assay using
121 the Pikp NLRs and the effector variants in the same expression vectors. We obtained
122 equivalent results to those shown previously³³ (**Supplementary Fig. 1a,b**). The
123 expression of each protein was confirmed by western blot (**Supplementary Fig. 1c**).

124

125 **Allele-specific effector responses in planta correlates with direct Pik-HMA**
126 **interactions**

127 We used yeast-2-hybrid (Y2H) to investigate whether the binding of effectors to the
128 Pikp-HMA domain (henceforth Pikp-HMA) or Pikm-HMA domain (henceforth Pikm-
129 HMA) correlates with in planta response profiles. We observed comparable growth of
130 yeast on selective plates, and the development of blue colouration with X- α -gal (both
131 indicative of protein/protein interactions), with Pikm-HMA and AVR-PikD, AVR-
132 PikE, and AVR-PikA, but not AVR-PikC (**Fig. 2a**). While the Y2H assay with Pikm-
133 HMA or Pikp-HMA showed comparable interaction with AVR-PikD, Pikm-HMA
134 showed increased interaction with AVR-PikE and markedly stronger interaction with
135 AVR-PikA (**Fig. 2a**). No growth was observed with Pikp-HMA and AVR-PikC. All
136 proteins were confirmed to be expressed in yeast (**Supplementary Fig. 2a**).

137

138 **Pikm-HMA has tighter binding affinities for AVR-Pik effectors compared to Pikp-**
139 **HMA in vitro**

140 To produce stable Pikm-HMA protein for in vitro studies, we cloned a construct with
141 a 5-amino acid extension at the C-terminus (encompassing residues Gly186 - Asp264
142 of the full-length protein) compared to the previously studied Pikp-HMA³³. Using gel
143 filtration with separately purified proteins, Pikm-HMA forms complexes with the
144 effectors AVR-PikD, AVR-PikE, or AVR-PikA, but not with AVR-PikC (**Fig. 2b**,
145 **Supplementary Fig. 2b**).

146 To determine the extent to which the expanded response of Pikm to AVR-Pik effectors
147 in *N. benthamiana* is related to the strength of binding to the Pikm-HMA, we

148 determined binding affinities by Surface Plasmon Resonance (SPR). We monitored
149 response units (RU) following Pikm-HMA injection after capturing effectors on the
150 chip surface. Binding of Pikm-HMA to the different effectors was measured at three
151 different concentrations, and RUs normalised to R_{\max} (theoretical maximum response,
152 assuming a 1:1 interaction model). From this, we ranked the order of highest to lowest
153 apparent affinity (**Fig. 2c**). We then extended the Pikm-HMA concentration range to
154 enable estimation of the equilibrium dissociation constant, K_D . Using a 1:1 kinetics
155 interaction model, we found that Pikm-HMA bound to AVR-PikD with the highest
156 affinity (lowest K_D), followed by AVR-PikE and AVR-PikA (**Fig. 2c, Supplementary**
157 **Fig. 2c-e, Supplementary Table 1**). We observed no significant binding of Pikm-HMA
158 to AVR-PikC (**Fig. 2c, Supplementary Fig. 2f, Supplementary Table 1**).

159 We also produced Pikip-HMA with its equivalent 5-amino acid C-terminal extension
160 (including residues Gly186 – Asp263 of the full-length protein) and analysed effector
161 binding by SPR (**Fig. 2c**). We ranked effector binding affinities in the order AVR-PikD
162 > AVR-PikE > AVR-PikA (with no significant binding to AVR-PikC, and assuming a
163 1:2 (effector:Pikip-HMA) interaction model, as previously observed³³). However, we
164 were only able to reliably determine the K_D for Pikip-HMA bound to AVR-PikD (**Fig.**
165 **2c, Supplementary Fig. 2g**), as the binding of AVR-PikE and AVR-PikA were of
166 insufficient quality under our assay conditions to allow K_D s to be determined
167 (**Supplementary Fig. 2h-i**).

168 Based on these results, and the interactions monitored by Y2H, we conclude that
169 differential binding affinity to the HMA domains is the source of the allele-specific
170 response profile in *N. benthamiana*, and of rice cultivars to *M. oryzae* strains expressing
171 AVR-Pik variants³⁴.

172

173 **Structures of Pik-HMAs in complex with AVR-Pik effectors reveals multiple** 174 **interaction surfaces**

175 Using a co-expression strategy, we obtained complexes of Pikm-HMA bound to AVR-
176 PikD, AVR-PikE, or AVR-PikA. Each of these were crystallised, and X-ray diffraction
177 data were collected at the Diamond Light Source (UK) to 1.2 Å, 1.3 Å, and 1.3 Å
178 resolution respectively. Details of X-ray data collection, structure solution, and
179 structure completion are given in the **Methods** and **Supplementary Table 2**. The
180 overall orientations of each component in the Pikm-HMA/effector complexes are
181 similar to each other, and to the previously determined Pikip-HMA/AVR-PikD
182 structure³³ (**Fig. 3a, Supplementary Fig. 3a,b, Supplementary Table 3**). Interestingly,
183 the Pikm-HMA/effector structures form a 1:1 complex, in contrast to Pikip-
184 HMA/AVR-PikD, which formed a 2:1 complex³³. Pikip-HMA dimerization is most
185 likely an artefact of in vitro protein expression and purification.

186 Analysis of the interfaces formed between Pikm-HMA and the effectors using
187 QtPISA³⁶ (**Supplementary Table 4, Supplementary Fig. 4**) reveals they are broadly

188 similar to each other, although there is a trend of reducing total interface area in the
189 order AVR-PikD > AVR-PikE > AVR-PikA. Graphical representation of key interface
190 components (using QtPISA interaction radars³⁶, **Supplementary Fig. 4**) reveals a high
191 likelihood that each interface is biologically relevant: each key component value lies
192 well above the 50% threshold when considered against statistical distributions derived
193 from the Protein DataBank (PDB) (see **Methods** and ³⁶).

194 Three predominant regions can be identified within each Pikm-HMA/effector
195 interface (**Fig. 3b**, **Fig. 1c**). These regions (interfaces) are defined here from the HMA
196 side as: interface 1, N-terminal residues Glu188 – Lys191; interface 2, residues from β 2
197 and β 3 (Ser219 – Val233), and Lys195 from β 1; interface 3, residues from β 4 to the C-
198 terminus (Met254 – Asp264) (**Fig. 3b**, **Fig. 1c**).

199 Interface 1 is a minor component of the Pikm-HMA/effector interaction, with a single,
200 weak hydrogen bond formed by the side-chain of Lys191 (to the main-chain carbonyl
201 group of Thr69 of the effector), and a hydrophobic interface contributed by the side
202 chain of Met189 (to the side chain of Ile49 of the effector). Interface 2 is more extensive,
203 and predominately interacts with AVR-Pik residues from the N-terminal extension of
204 the conserved MAX effector fold³⁷, including Arg39 – Phe44 and His46 – Ile49. This
205 interface includes the polymorphic residues at positions 46, 47, and 48 of the effector
206 variants³⁴ (**Fig. 1b**, **Fig. 3a-d**). Interface 2 also includes salt-bridge/hydrogen bond
207 interactions via the side chains of Asp225 (to Arg64 of the effectors), and Lys195 (to
208 Asp66 of the effectors, **Fig. 3a**). Finally, interface 3 includes both main-chain hydrogen
209 bonding interactions between β 4 of the HMA and β 3 of the effectors, and inserts the
210 side-chain of Lys262 into a surface pocket on the effector lined by residues Glu53,
211 Tyr71, Ser72, and Trp74. Lys262 makes a number of interactions in this pocket,
212 including salt-bridge/hydrogen bonds with the side-chains of Glu53 and Ser72 (**Fig.**
213 **3a**, **Fig. 4a**).

214 We also obtained crystal structures of Pikip-HMA, with the 5-amino acid extension at
215 the C-terminus of the HMA, bound to AVR-PikD or AVR-PikE at 1.35 Å and 1.9 Å
216 resolution respectively (see **Methods**, **Supplementary Table 2**, **Supplementary Fig.**
217 **3c,d**). The Pikip/AVR-PikE combination does not give rise to responses in planta, but
218 we were able to obtain the complex in solution. The new structure of the Pikip-
219 HMA/AVR-PikD complex is essentially identical to that previously determined³³,
220 except for the 5-amino acid extension. Interface analysis with QtPISA (**Supplementary**
221 **Table 4**, **Supplementary Fig. 4**) reveals that the Pikip-HMA/AVR-PikD complex has
222 broadly similar properties to those of Pikm-HMA/effectors (total interface area and
223 key component values well above the 50% threshold in interaction radars). In contrast,
224 while the Pikip-HMA/AVR-PikE interface shows a broadly similar total interface area
225 to the other complexes, the total calculated binding energy is reduced (area of the
226 polygon in **Supplementary Fig. 4**), and 5 out of 6 key interface components fall below
227 the 50% threshold, questioning this interface's biological relevance.

228

229 **Structural changes at interface 2 underpin differential effector recognition by Pikm**

230 Effector variants AVR-PikD, AVR-PikE, and AVR-PikA differ at amino acid positions
231 46, 47 and 48, which localise to interface 2 (**Fig. 1b, Fig. 3b**). Pikp-HMA binds AVR-
232 PikD(His46) via hydrogen bonds with residues Ser218 and Glu230³³. In Pikm, the Ser
233 is conserved, but Glu230 is replaced by Val231 at the structurally equivalent position,
234 resulting in the loss of a direct hydrogen bond. Despite this, AVR-PikD(His46)
235 occupies the same position in both complexes (**Fig. 3c**). Surprisingly, in the Pikm-
236 HMA/AVR-PikE complex, AVR-PikE(Asn46) is rotated out of the binding pocket,
237 well away from Val231 (**Fig. 3d**), and a water molecule occupies the resulting space.
238 Hydrogen bonds are formed between AVR-PikE(Asn46:N δ 2) and both Pikm-
239 HMA(Ser219:OH) and the new water molecule. This configuration impacts the
240 position of effector residues Phe44 – Gly48, pushing them away from the HMA, further
241 altering interactions across interface 2. These structural changes correlate with reduced
242 binding affinity of AVR-PikE with Pikm-HMA compared to AVR-PikD. In the Pikm-
243 HMA/AVR-PikA complex, Asn46 is rotated even further out of the HMA pocket, and
244 while a hydrogen bond is still formed with Pikm-HMA(Ser219:OH), this is
245 significantly different in orientation (**Fig. 3d**). These changes serve to move residues
246 Asn46 – Pro50 of AVR-PikA further away from the HMA, and again these structural
247 observations correlate with reduced effector binding affinity. Interestingly, the
248 polymorphic residues in AVR-PikA (Ala47 and Asp48) have no direct role in Pikm-
249 HMA interaction. The polymorphisms in AVR-Pik do not significantly alter
250 protein/protein interactions across interfaces 1 and 3, and these regions appear to
251 stabilise the complexes.

252 We conclude that the structural changes at interface 2 underlie the weaker binding
253 affinities of Pikm-HMA for AVR-PikE and AVR-PikA, compared to AVR-PikD.

254

255 **Interactions across interface 3 contribute more to Pikm-HMA than Pikp-HMA**
256 **binding to AVR-PikD**

257 As observed at interface 3 for the Pikm/effector complexes (**Fig. 4a**), a Lys residue
258 from Pikp-HMA (Lys262) locates to the binding pocket on the effector containing
259 Glu53 and Ser72 (**Fig. 4b**). However, this Lys is shifted one residue to the C-terminus
260 in the sequence of Pikp-1 (**Fig. 1c**). This results in a different conformation of Pikp-
261 HMA residues Ala260 and Asn261 when compared to Pikm-HMA (Val261 and
262 Lys262), changing the interactions across interface 3. The most dramatic difference is
263 the “looping-out” of Pikp-HMA(Asn261), to retain Lys262 in the effector binding
264 pocket (**Fig. 4b, Fig. 5d,e**), which affects the packing of Pikp-HMA(Ala260) (Val261 in
265 Pikm-HMA) and hydrophobic packing of the side-chain of Lys262.

266 Pik alleles also differ in the composition of residues at interfaces 1 and 2. Of most
267 significance are the changes at interface 2 that contact AVR-PikD(His46), as discussed
268 above and **Fig. 3c**.

269 We propose that Pikm has evolved more robust interactions across interface 3
270 compared to Pikp to compensate for loss of binding, such as direct hydrogen bonds,
271 at interface 2.

272

273 **Interactions across interfaces 2 and 3 underpin specificity of Pikp to AVR-PikD over** 274 **AVR-PikE**

275 Underpinning the global analysis of the Pikp-HMA/AVR-PikD and Pikp-
276 HMA/AVR-PikE complexes are extensive differences at interfaces 2 and 3. At interface
277 2, AVR-PikE(Asn46) is fully rotated out of the AVR-PikD(His46) binding pocket (**Fig.**
278 **5a-c**). A hydrogen bond is still formed between AVR-PikE(Asn46) and Pikp-
279 HMA(Ser218), but in a very different orientation (**Fig. 5a-c**). This results in residues
280 Asn46-Pro50 moving away from the HMA. This re-configuration is coupled with
281 changes at interface 3 (**Fig. 5d,f,g**). Interestingly, in the Pikp-HMA/AVR-PikE
282 complex, Lys262 adopts a similar orientation to that found in the Pikm-HMA
283 complexes (**Fig. 5e,f,g**). But to enable this, residues Ser258 - Asn261 adopts a
284 dramatically different position, looping-out residues Gln259 and Ala260 from their
285 positions in the Pikm-HMA complex (**Fig. 5e,f,g**), with consequent impacts on this
286 interface.

287 We conclude that interface 2 is key for effector recognition by Pikp and, unlike for
288 Pikm, interfaces 1 and 3 are not able to compensate to enable productive binding.

289

290 **Mutations at separate interfaces have differential effects on Pik-HMA/effector** 291 **interactions and immunity phenotypes**

292 We subsequently tested whether mutations in the effectors at interfaces 2 and 3 have
293 differential effects on Pik-HMA binding and responses by Y2H, SPR and in *N.*
294 *benthamiana*. We used the previously characterised AVR-PikD(His46Glu) mutant at
295 interface 2, and a Glu53Arg mutant at interface 3 in AVR-PikD, AVR-PikE, and AVR-
296 PikA. While AVR-PikD(His46) occupies a central position at interface 2, AVR-
297 Pik(Glu53) locates to the Pik-HMA(Lys262) binding pocket, at the periphery of
298 interface 3.

299 As previously observed (although without the C-terminal extension³³), the AVR-
300 PikD(His46Glu) mutant essentially blocks the Pikp-HMA/effector interaction in Y2H
301 and SPR, and abolishes Pikp-mediated cell death in *N. benthamiana* (**Fig. 6a-c,**
302 **Supplementary Fig. 5**). Interestingly, AVR-PikD(His46Glu) interacts with Pikm-HMA
303 in Y2H (**Fig. 6a**). However, when measured by SPR, Pikm-HMA binding to this mutant
304 is reduced to ~11% compared to wild-type (**Fig. 6b**). This reduction of binding in vitro
305 is reflected in *N. benthamiana*, where we observe weak AVR-PikD(His46Glu)-
306 dependent Pikm cell death (**Fig. 6c, Supplementary Fig. 5b-d**).

307 For each of the Glu53Arg effector mutants, we observe little impact on Pikm-HMA
308 interaction in Y2H compared to wild-type, except a reduced interaction of AVR-
309 PikA(Glu53Arg) (**Fig. 6a**). Interestingly, the Glu53Arg mutant in AVR-PikE abolishes
310 interaction of this effector with Pikp-HMA in Y2H. Using SPR, the AVR-
311 Pik(Glu53Arg) mutants show reduced binding to both Pik-HMA domains when
312 compared pairwise with wild-type in each effector background (**Fig. 6b**). However, in
313 each case, the Glu53Arg mutant has a greater effect in Pikm-HMA binding compared
314 to Pikp-HMA. Surprisingly, in the *N. benthamiana* cell death assay, we observe a slight
315 increase in the AVR-PikD(Glu53Arg)-dependent cell death compared to wild-type for
316 both Pikp and Pikm (**Fig. 6c, Supplementary Fig. 5b-d**). However, we see a reduction
317 in intensity of Pikm-mediated cell death for the effector variants AVR-PikE(Glu53Arg)
318 and AVR-PikA(Glu53Arg) (**Fig. 6c, Supplementary Fig. 5b-d**).

319 We conclude that interactions across interface 2 are critical for effector recognition by
320 Pikp, and important for Pikm, and interface 3 has an important role in the extended
321 response of Pikm to AVR-PikE and AVR-PikA.

322 Discussion

323 Despite intensive study, 25 years since the cloning of the first plant NLRs³⁸⁻⁴⁰ very little
324 is known about the molecular mechanistic basis of how these proteins recognise
325 pathogen effectors and initiate immune signalling. The recent identification of plant
326 NLRs with integrated domains²⁸⁻³⁰ has enabled new opportunities to investigate how
327 these receptors directly recognise pathogen effectors at the biochemical and structural
328 level, and how these binding events are linked to disease resistance^{33,41-44}. Here we
329 have generated five structures of different complexes between the integrated domains
330 of an allelic NLR (Pik), and the variants of the effector (AVR-Pik) they recognise. When
331 combined with analysis of biophysical interactions in vitro, and cell death responses
332 in the model plant *N. benthamiana*, these structures provide new understanding, and
333 unexpected findings, on how co-evolution has driven the emergence of new plant NLR
334 receptor specificities.

335 High levels of diversifying selection in allelic plant NLRs and pathogen effectors
336 suggest direct interaction between the proteins. Previous studies where structures of
337 the effectors, but not the interacting NLR domain, were available showed that
338 distributed surface-presented residues on the effectors defined NLR recognition
339 specificity, mediated by polymorphic LRR domains^{14,15}. The integrated HMA domains
340 are the most polymorphic regions of the rice Pik-1/Pik-2 paired NLRs, and Pik-HMA
341 amino acids that form the interfaces with effectors are likely under the strongest
342 selective pressure. Therefore, during the course of plant-pathogen co-evolution, at
343 least two alternative solutions for recognising divergent effectors have emerged. One
344 of these involves the integration and diversification of non-canonical domains in the
345 NLR architecture. The second involves diversification of LRR domains. An important
346 question raised by these studies is what has driven the emergence of these different
347 systems? An advantage of the integrated domain is that (once stably incorporated) it
348 may tolerate accelerated accumulation of mutations, followed by selection for
349 function, as mutations may be less likely to disrupt to the overall structure and
350 function of the NLRs.

351 One outcome from this work is the surprising plasticity of the Pik-HMA interfaces that
352 supports differential recognition of AVR-Pik variants. Interactions across interface 2
353 are important for effector binding by Pikp-HMA and Pikm-HMA. Disruption of
354 interface 2 by amino acid polymorphisms in AVR-PikE and AVR-PikA eliminates
355 Pikp-mediated cell death in planta, and weakens Pikm-mediated cell death. The
356 unique polymorphism that defines AVR-PikC (Ala67Asp) also maps to interface 2, and
357 may result in a steric clash preventing, or severely reducing, Pik-HMA binding. Our
358 structural data support a conclusion that more favourable interactions across interface
359 3 have evolved in Pikm-HMA to, in-part, compensate for the impact of AVR-Pik
360 variation at interface 2, and support cell-death signalling. Our biophysical data suggest
361 that quantitative binding differences, visualised as disruption of interfaces in the
362 structures, underpins differential effector recognition by Pik-HMAs, and a threshold
363 of binding is required for activation of response in planta. These insights will inform

364 future structure/function studies to address whether rational engineering of Pik-
365 HMA effector-binding interfaces can generate NLR receptors with improved
366 recognition profiles. Ultimately, we must understand how recognition of effectors,
367 through either integrated domains or other mechanisms, results in triggering of
368 immune responses in the context of the full-length proteins and, potentially,
369 oligomeric states.

370 **Methods**

371 **Gene cloning**

372 For details of gene cloning, please see **Supplementary Methods**.

373 **Expression and purification of proteins for in vitro binding studies**

374 pOPINM encoding Pikm-HMA or Pikp-HMA was transformed into *E. coli* SHuffle
375 cells⁴⁵. Inoculated cell cultures were grown in auto induction media⁴⁶ at 30 °C for 6hr
376 and 18 °C overnight. Cells were harvested and proteins extracted as previously
377 reported³³. AVR-Pik effectors with a cleavable N-terminal SUMO or MBP tag and a
378 non-cleavable C-terminal 6xHis tag were produced in and purified from *E. coli* SHuffle
379 cells as previously described³³ using either auto induction media⁴⁶, or Power Broth
380 (Molecular Dimensions).

381 Protein concentration of AVR-Pik effectors was determined by absorption at 280 nm
382 using a NanoVue spectrophotometer (GE Lifesciences). Measurements were corrected
383 using the molar extinction coefficient 25,105 M⁻¹ cm⁻¹, as calculated by Expasy
384 (<http://web.expasy.org/protparam>). Due the lack of aromatic residues in Pik-HMA
385 domains, protein concentrations were measured using a Direct Detect® Infrared
386 Spectrometer (Merck).

387 **Co-expression and purification of Pik-HMA/AVR-Pik effectors for crystallisation.**

388 Relevant Pik-HMA domains and AVR-Pik effectors were co-expressed in SHuffle cells
389 following co-transformation of pOPINM:Pik-HMA and pOPINA:AVR-Pik, as
390 previously described³³. Cells were grown in autoinduction media (supplemented with
391 both carbenicillin and kanamycin), harvested, and processed as described in the
392 **Supplementary Methods**. Protein concentrations were measured by absorbance at 280
393 nm using a NanoVue spectrophotometer and an extinction coefficient of 25,105 M⁻¹ cm⁻¹
394 ¹ for Pikm-HMA complexes, and 26,720 M⁻¹ cm⁻¹ for Pikp-HMA complexes, as
395 calculated by Expasy (<http://web.expasy.org/protparam>).

396 **Protein:protein interaction: Analytical gel filtration**

397 Pikm-HMA and the AVR-Pik effectors were mixed in a molar ratio of 2:1 and
398 incubated on ice for 60 min. In each case a sample volume of 110 µl was separated at
399 4°C on a Superdex 75 10/300 size exclusion column (GE Healthcare), pre-equilibrated
400 in buffer B, and at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected for
401 analysis by SDS-PAGE. The Superdex 75 10/300 column has a void volume of 7.4 ml
402 and a total volume of 24 ml.

403 **Protein:protein interaction: Surface plasmon resonance**

404 Surface plasmon resonance (SPR) experiments were performed on a Biacore T200
405 system (GE Healthcare) using an NTA sensor chip (GE Healthcare). All proteins were
406 prepared in SPR running buffer (20 mM HEPES pH 7.5, 860 mM NaCl, 0.1% Tween

407 20). Details of the cycling conditions are given in the **Supplementary Methods**.

408 The equilibrium dissociation constants (K_D) for Pikm-HMA binding to AVR-Pik
409 alleles, and Pikip-HMA binding to AVR-PikD, were determined from multicycle
410 kinetics curves using the Biacore T200 BiaEvaluation software (GE Healthcare), with a
411 1:1 or 2:1 fit model respectively. For the interaction between Pikip-HMA and AVR-PikE
412 and AVR-PikA, and for both Pik-HMAs and the AVR-Pik mutants, it was not possible
413 to accurately determine the K_D due to the insufficient quality of the data. In these cases,
414 the level of binding was expressed as a percentage of the theoretical maximum
415 response (R_{max}) normalized for the amount of ligand immobilized on the chip. SPR
416 data was exported and plotted using Microsoft Excel. Each experiment was repeated
417 a minimum of 3 times, with similar results.

418 **Protein:protein interaction: Yeast-2-hybrid analyses**

419 The Matchmaker® Gold Yeast Two-Hybrid System (Takara Bio USA) was used to
420 detect protein–protein interactions between Pik-HMAs and AVR-Pik effectors. DNA
421 encoding Pik-HMAs in pGBKT7 was co-transformed with either the individual AVR-
422 Pik variants or mutants in pGADT7, into chemically competent *Saccharomyces cerevisiae*
423 Y2HGold cells (Takara Bio, USA). Single colonies grown on selection plates were
424 inoculated in 5 ml of SD^{-Leu-Trp} and grown overnight at 30°C. Saturated culture was
425 then used to make serial dilutions of OD₆₀₀ 1, 1⁻¹, 1⁻², 1⁻³, respectively. Five µl of each
426 dilution was then spotted on a SD^{-Leu-Trp} plate as a growth control, and also on a SD<sup>-Leu-
427 Trp-Ade-His</sup> plate containing X-α-gal and aureobasidine, as detailed in the user manual.
428 Plates were imaged after incubation for 60 - 72 hr at 30 °C. Each experiment was
429 repeated a minimum of 3 times, with similar results.

430 To confirm protein expression in yeast, total protein was extracted from transformed
431 colonies by boiling the cells for 10 minutes in LDS Runblue® sample buffer. Samples
432 were centrifugated and the supernatant was subjected to SDS-PAGE prior to western
433 blotting. The resulting membranes were probed with Anti-GAL4 DNA-BD (Sigma) for
434 HMA domains in pGBKT7 and Anti-GAL4 Activation Domain (Sigma) antibodies for
435 AVR-Pik effectors in pGADT7.

436 ***N. benthamiana* cell death assays**

437 Transient gene-expression in planta was performed by delivering T-DNA constructs
438 with *Agrobacterium tumefaciens* GV3101 strain into 4-week old *N. benthamiana* plants
439 grown at 22–25 °C with high light intensity. Pik-1, Pik-2, AVR-Pik and P19 were mixed
440 at OD₆₀₀ 0.4, 0.4, 0.6 and 0.1, respectively. Detached leaves were imaged at 5 dpi from
441 the abaxial side. Images are representative of three independent experiments, with
442 internal repeats. The cell death index used for scoring is as presented previously³³ (also
443 included in **Supplementary Fig. 1d**). Scoring for all replicas is presented as boxplots,
444 generated using R v3.4.3 (<https://www.r-project.org/>) and the graphic package
445 ggplot2⁴⁷. The centre line represents the median, box limits are upper and lower

446 quartiles, whiskers are 1.5x interquartile range, and all data points are represented as
447 dots.

448 The presence of each protein, as expressed in representative assays, was determined
449 by SDS-PAGE/western blot. For this, leaf tissue was frozen, and ground to fine
450 powder in liquid nitrogen using a pestle and mortar. Leaf powder was mixed with 2
451 times weight/volume ice-cold extraction buffer (10% glycerol, 25 mM Tris pH 7.5, 1
452 mM EDTA, 150 mM NaCl, 2% w/v PVPP, 10 mM DTT, 1x protease inhibitor cocktail
453 (Sigma), 0.1% Tween 20 (Sigma)), centrifuged at 4,200g/4 °C for 20-30 min, and the
454 supernatant filtered (0.45 µm).

455 **Crystallization, data collection and structure solution**

456 For crystallization, Pik-HMA/AVR-Pik complexes were concentrated in buffer B (see
457 **Supplementary Methods**). Sitting drop, vapor diffusion crystallization trials were set
458 up in 96 well plates, using an Oryx nano robot (Douglas Instruments, United
459 Kingdom). Plates were incubated at 20°C, and crystals typically appeared after 24 - 48
460 hours. For data collection, all crystals were harvested from the Morpheus® HT-96
461 screen (Molecular Dimensions), and snap-frozen in liquid nitrogen. Details of each
462 crystallisation condition are given in the **Supplementary Methods**.

463 X-ray data sets were collected at the Diamond Light Source (Oxford, UK). The data
464 were processed using the xia2 pipeline⁴⁸ and AIMLESS⁴⁹, as implemented in CCP4⁵⁰.
465 The structures were solved by molecular replacement using PHASER⁵¹ and the Pikp-
466 HMA/AVR-PikD structure³³. The final structures were obtained through iterative
467 cycles of manual rebuilding and refinement using COOT⁵² and REFMAC5⁵³, as
468 implemented in CCP4⁵⁰. Structures were validated using the tools provided in COOT
469 and MOLPROBITY⁵⁴.

470 **Protein interface analyses**

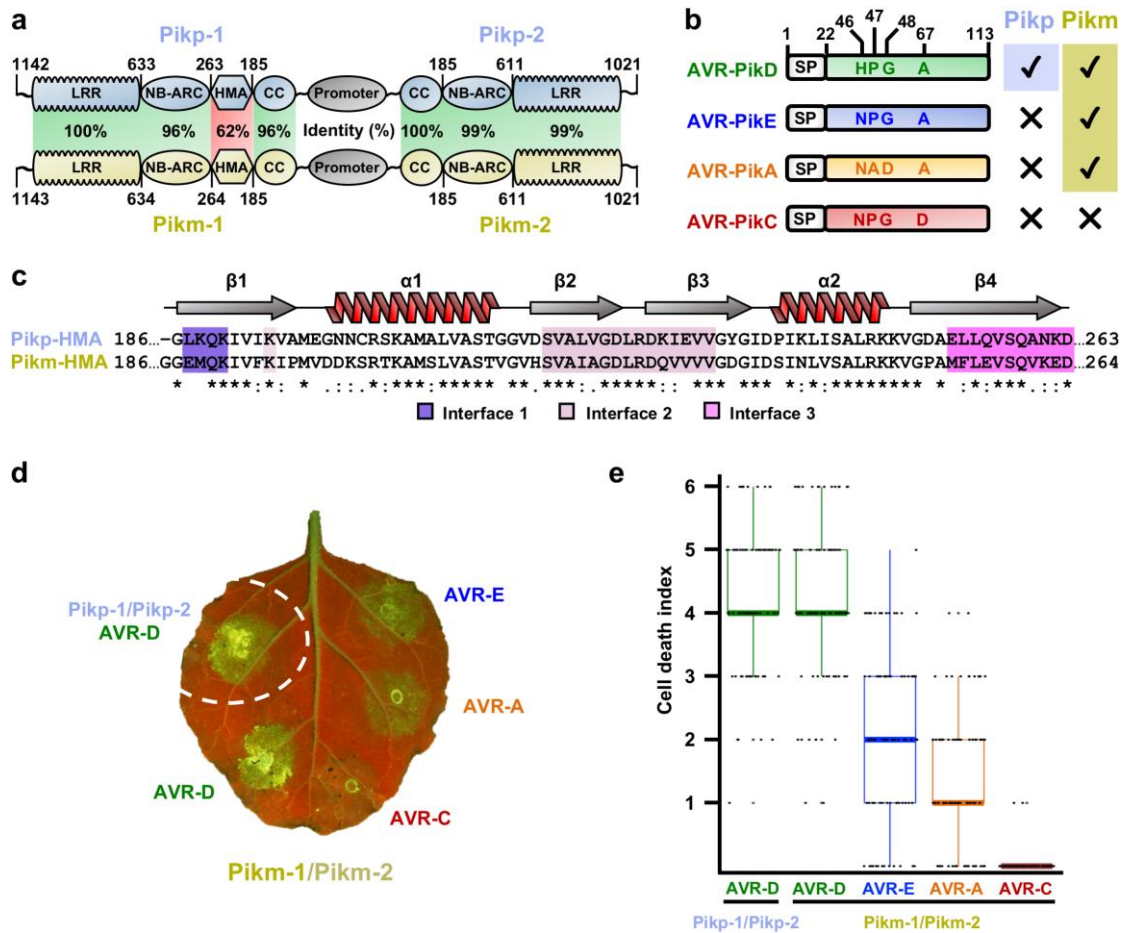
471 Protein interface analyses were performed using QtPISA³⁶. For each complex, one Pik-
472 HMA/AVR-Pik effector assembly was used as a representative example. QtPISA
473 interaction radars³⁶ were produced using the reference parameter “Total Binding
474 Energy”. The area of the polygon indicates the likelihood of the interface to constitute
475 part of a biological assembly (the greater the area the more likely). The scales along the
476 beams compare key interface properties to statistical distributions derived from the
477 Protein Databank. In general, if the radar area is contained within the 50% probability
478 circle then the interface is considered superficial, and its biological relevance is
479 questionable. In cases where the radar area is expanded outside the 50% probability
480 circle, the interface is considered more likely to be significant and biologically
481 relevant³⁶.

482 **Data availability**

483 The co-ordinates and structure factors have been deposited in the Protein Data Bank
484 with accession codes 6FU9 (Pikm-HMA/AVR-PikD), 6FUB (Pikm-HMA/AVR-PikE),
485 6FUD (Pikm-HMA/AVR-PikA), 6G10 (Pikp-HMA/AVR-PikD) and 6G11 (Pikp-
486 HMA/AVR-PikE).

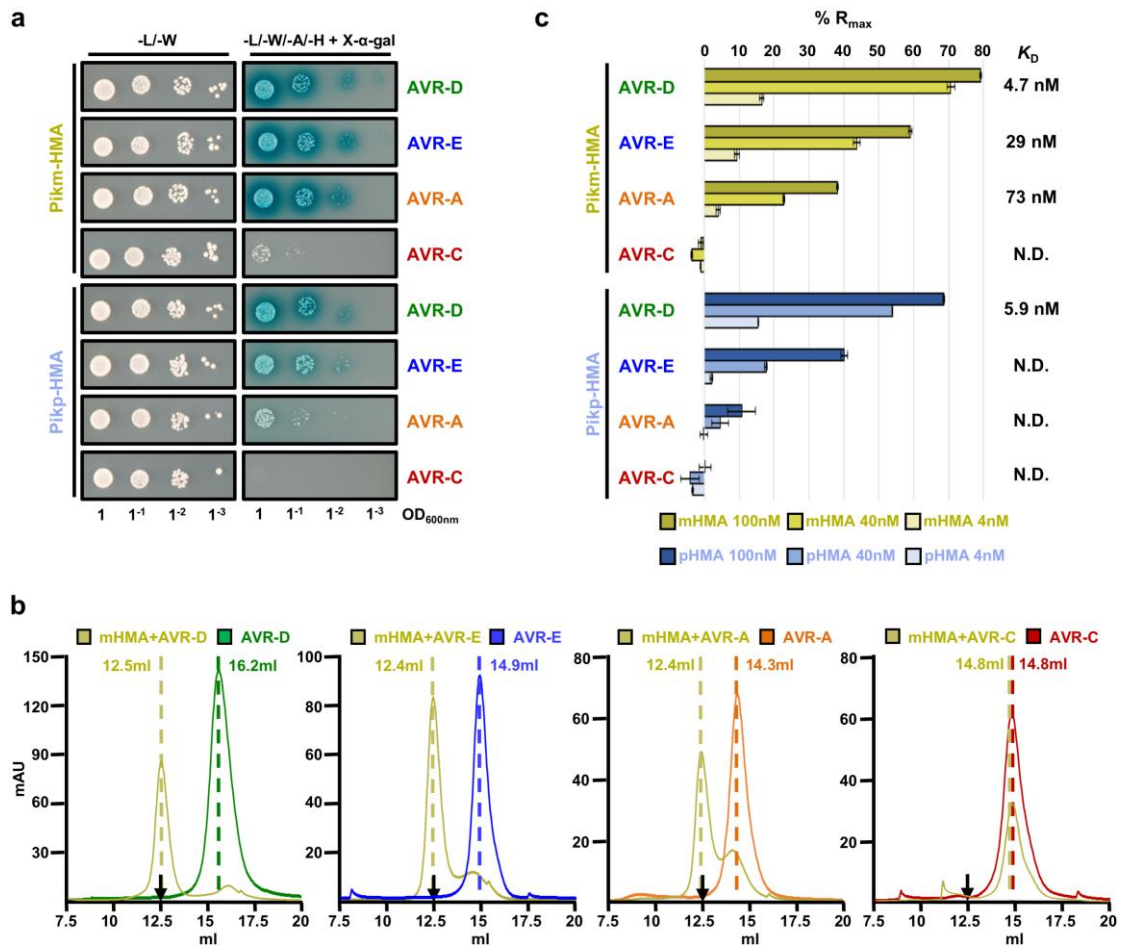
487 **Acknowledgements**

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492 to X-ray data collection facilities. We also thank David Lawson and Clare Stevenson
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494 structure determination and SPR.



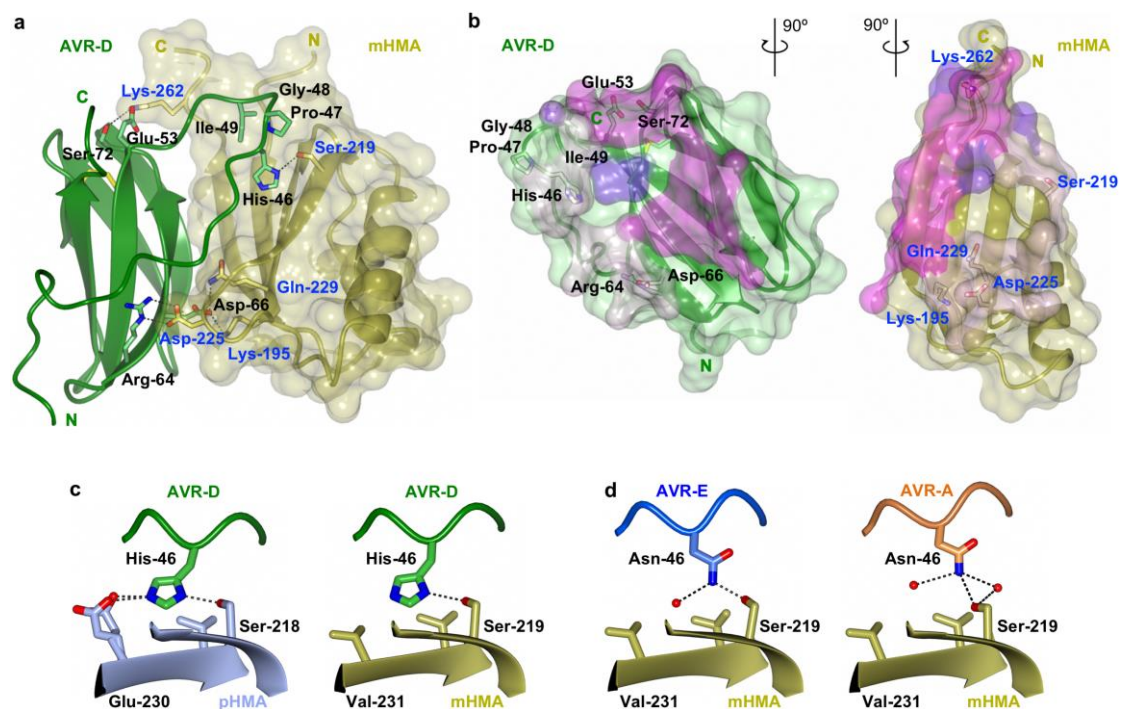
496

497 **Figure 1: The Pikm-mediated cell death response to AVR-Pik effector variants in *N.***
 498 ***benthamiana* phenocopies the Pikm resistance profile in rice. (a)** Schematic
 499 representations of Pik NLR alleles. The sensor NLR (Pik-1) and helper NLR (Pik-2)
 500 share a common promoter and the same overall domain architecture. Pikp-1/Pikp-2
 501 (top) are shown in ice blue, and Pikm-1/Pikm-2 (bottom) are shown in gold. Pairwise
 502 protein sequence identity between each domain is indicated, highlighting
 503 diversification of the integrated HMA domain, **(b)** Schematic representations of AVR-
 504 Pik variants with amino acid polymorphisms shown (single letter code, SP = Signal
 505 Peptide), along with their Pikp- or Pikm-mediated response profiles in rice³⁴, **(c)**
 506 Amino acid sequence alignment of Pikp-1 and Pikm-1 HMA domains. Secondary
 507 structure features of the HMA fold are shown above, and the residues located to the
 508 interfaces described in the text and **Figure 3** are highlighted in purple (interface 1),
 509 pink (interface 2), and magenta (interface 3), **(d)** Representative leaf image showing
 510 Pikm-mediated cell death to AVR-Pik variants as autofluorescence under UV-light,
 511 Pikp-mediated cell death with AVR-PikD is included as a positive control (surrounded
 512 by dashed circle, no Pikm-1/Pikm-2 in this spot), **(e)** Box-plots showing repeats of the
 513 cell death assay, for each sample the number of repeats was 90. The cell-death scoring
 514 scale used is shown in **Supplementary Fig. 1d**. For brevity, effectors are labelled
 515 without the 'Pik' designation in panels **(d)** and **(e)** and, where appropriate, in **Figs. 2 -**
 516 **6.**



518

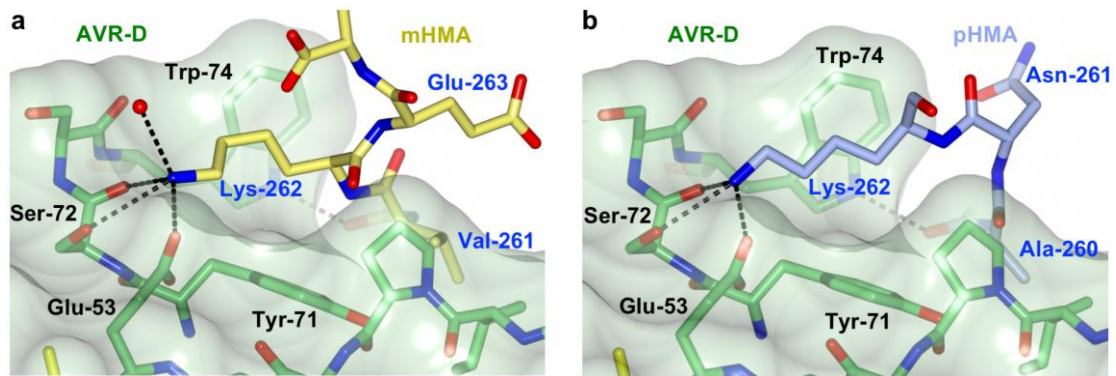
519 **Figure 2: Different affinities underpin recognition and response of Pik NLR alleles**
 520 **to AVR-Pik effector variants. (a)** Yeast-2-hybrid demonstrates binding of effector
 521 variants to both the Pikm- and Pikp-HMA domains, control plate for yeast growth is
 522 on the left, with selective plate on the right, **(b)** Analytical gel filtration confirms that
 523 Pikm-HMA forms complexes with AVR-PikD, AVR-PikE, and AVR-PikA in vitro, but
 524 not AVR-PikC. Note that earlier elution correlates with increased molecular mass.
 525 Retention volumes for peaks are labelled (black arrow indicates Pikm-HMA elution
 526 volume, Pikm-HMA does not absorb light at 280 nm). SDS-PAGE with relevant
 527 fractions are shown in **Supplementary Fig. 2b. (c)** Surface Plasmon Resonance (SPR)
 528 reveals in vitro binding affinity between Pik-HMA domains and effectors correlates
 529 with in planta responses. %R_{max} is the percentage of the theoretical maximum
 530 response, assuming a 1:1 binding model for Pikm (effector:HMA), and a 1:2
 531 binding model for Pikp, at the HMA concentrations shown. Bars represent the average of 3
 532 measurements, with corresponding standard deviation. Where K_D values are given, a
 533 wider range of HMA concentrations were used for this calculation (see
 534 **Supplementary Fig. 2c-e, g**), N.D. = Not Determined.



536

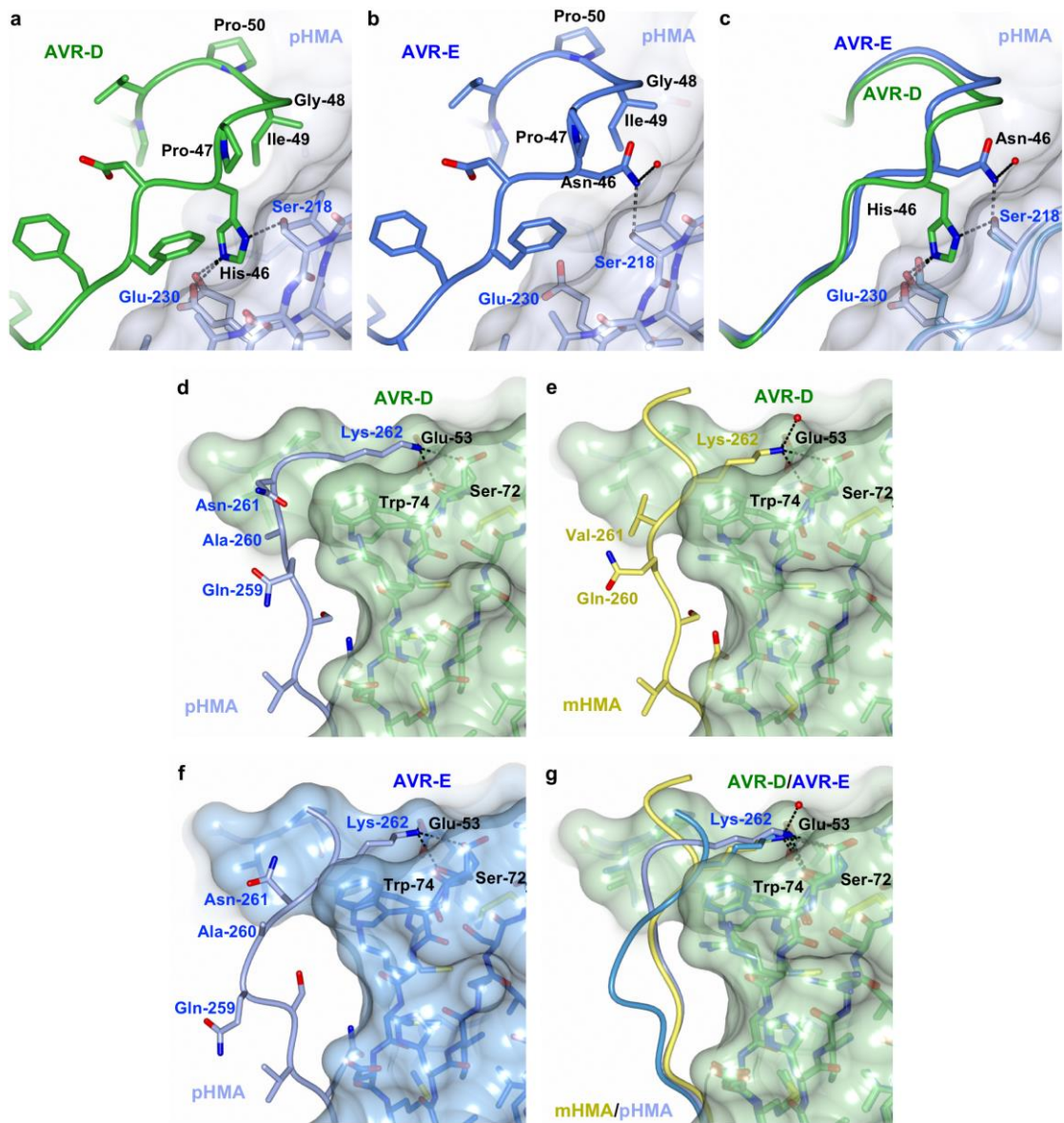
537 **Figure 3: Structures of Pikm-HMA in complex with AVR-Pik effectors.** (a) Schematic
 538 representation of the structure of Pikm-HMA in complex with AVR-PikD. Pikm-HMA
 539 is shown in gold cartoon representation with selected side chains as sticks; the
 540 molecular surface of this domain is also shown. AVR-PikD is shown in green cartoon,
 541 with selected side chains as sticks. Hydrogen bonds/salt bridges are shown as dashed
 542 lines and the di-sulfide bond as yellow bars, (b) Buried surface area of AVR-PikD and
 543 Pikm-HMA shown from the perspective of the partner (change in orientation from
 544 panel (a) indicated). The buried surfaces are coloured according to interfaces described
 545 in the text (interface 1 is in purple, interface 2 is in pink, interface 3 is magenta), (c)
 546 Close-up views (part of interface 2) of the orientation and interactions of AVR-
 547 PikD(His46) in the Pikp-HMA and Pikm-HMA complexes, (d) Close-up views (part of
 548 interface 2) of the orientation and interactions of AVR-PikE(Asn46), left, and AVR-
 549 PikA(Asn46), right, in complex with Pikm-HMA. Water molecules are shown as red
 550 spheres.

551



552

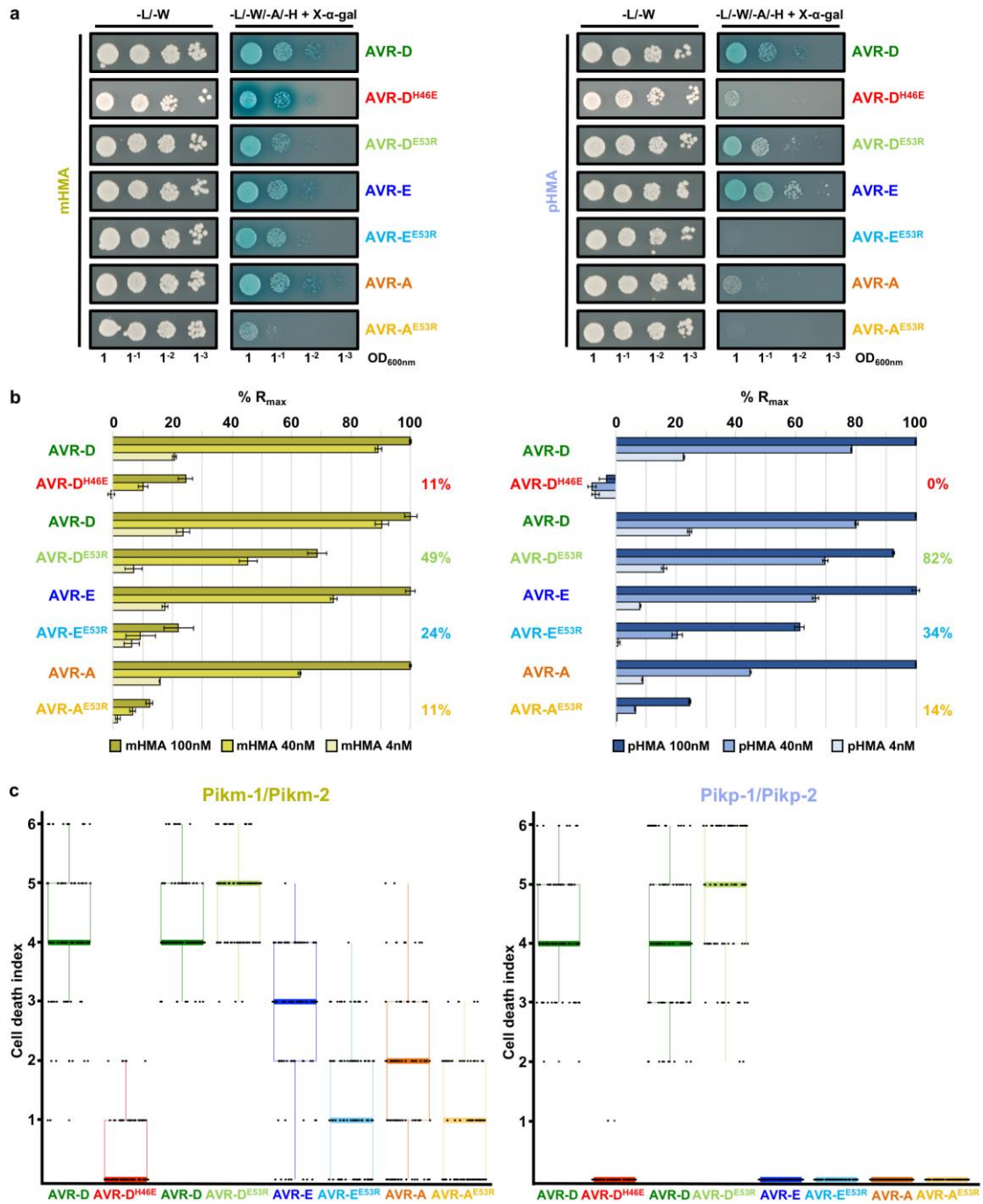
553 **Figure 4: Different interactions at interface 3 in the complexes of Pikm-HMA and**
554 **Pikp-HMA with AVR-PikD support recognition and response.** Close-up view of the
555 interactions across interface 3 in the (a) Pikm-HMA and (b) Pikp-HMA complexes with
556 AVR-PikD, showing different conformations for the C-terminal regions of the HMA
557 domains. In particular, note the looping-out of Asn261 of Pikp-HMA, and the different
558 orientation of the Lys262 sidechain. In each panel, AVR-PikD is shown in green
559 cartoon, with side chains as sticks, the molecular surface of the effector is also shown.
560 The Pik-HMA domains are coloured as labelled.



562

563 **Figure 5: Altered interactions across interfaces of Pikp-HMA with AVR-PikD and**
 564 **with AVR-PikE underpin differences in recognition and response. (a, b) Zoom-in**
 565 **views of the interactions across interface 2 in the Pikp-HMA complexes with AVR-**
 566 **PikD and AVR-PikE. In each panel the Pikp-HMA domain is shown as ice-blue sticks,**
 567 **the molecular surface is also shown. Effector variant residues are coloured as labelled**
 568 **and shown in α -worm with sidechain representation, (c) Superposition of panels (a)**
 569 **and (b), with only selected sidechains shown for clarity. The polymorphism at position**
 570 **46 occupies a very different position, fully flipped out of the His46 binding pocket in**
 571 **the AVR-PikE structure, which alters the position of residues Asn44-Pro50 relative to**
 572 **the Pikp-HMA domain, (d-f) Zoom-in views of the interactions across interface 3 in**
 573 **the Pikp-HMA complex with AVR-PikD, Pikm-HMA complex with AVR-PikD, and**
 574 **Pikp-HMA with AVR-PikE. In each panel the effector is shown as sticks, and the**
 575 **molecular surface is also shown and coloured as labelled. Pik-HMA residues are**
 576 **coloured as labelled and shown in α -worm with sidechain representation. The**

577 looping-out of Asn261 in Pikp compared to Pikm, when in complex with AVR-PikD,
578 is seen in panels **d** and **e**, and the displacement of residues Gln259 and Ala260 in Pikp,
579 between the complexes with AVR-PikD or AVR-PikE, is seen in panels **d** and **f**, **(g)**
580 Superposition of panels **(d-f)**, with only the sidechain of Pik-HMA Lys262, and only
581 the surface of AVR-PikD, shown for clarity.



583

584 **Figure 6: Mutations at different interfaces in the Pik-HMA/effector complexes have**
 585 **differential effects on interactions and phenotypes. (a)** Effector mutations at
 586 positions 46 and 53 perturb interactions with Pikm- and Pikip-HMA domains as
 587 assayed by Y2H, **(b)** Changes in in vitro binding for effector mutants with Pikm- and
 588 Pikip-HMA domains, as measured by SPR. %R_{max} was calculated as described in the
 589 text. To emphasise the altered binding for each effector mutant, the averaged
 590 difference % R_{max}, across the 3 different concentrations measured, is shown. Bars
 591 represent the average of 3 measurements, with corresponding standard deviation **(c)**

592 Box-plots of Pikm- or Pikp-mediated cell death triggered by the effector mutants, for
593 each sample the number of repeats was 90.

594 **Table 1: Summary Table detailing the various interactions and phenotypes**
 595 **between Pik NLR alleles and effector variants in this study.**

		AVR-D	AVR-E	AVR-A	AVR-C	AVR-D ^{H46E}	AVR-D ^{E53R}	AVR-E ^{E53R}	AVR-A ^{E53R}
Interaction in Y2H	Pikp	+++	++	+	-	+	++	-	-
	Pikm	+++	+++	+++	+	+++	++	++	+
Interaction in SPR	Pikp	+++	++	+	-	-	+++	+	-
	Pikm	+++	+++	++	-	+	++	+	-/+
Recognition in rice plants	Pikp	+++*	+*	(-)	(-)	-*	N.D.	N.D.	N.D.
	Pikm	(+++)	(+++)	(+++)	(-)	N.D.	N.D.	N.D.	N.D.
CD response in <i>N. benthamiana</i>	Pikp	+++	-	-	-	-	+++	-	-
	Pikm	+++	++	+	-	+	+++	+	+

596 Y2H = yeast-2-hybrid, SPR = Surface Plasmon Resonance, Recognition in rice plant Pikp = rice cv. K60, Pikm= rice cv.
 597 Tsuyuake, CD = cell death, N.D. not determined, parenthesis from³⁴, *from³³. SPR and Y2H interactions used the
 598 isolated HMA domains, *in planta* experiments were performed with full length proteins.

599 **References**

- 600 1 Jones, J. D., Vance, R. E. & Dangl, J. L. Intracellular innate immune surveillance devices
601 in plants and animals. *Science* **354**, doi:10.1126/science.aaf6395 (2016).
- 602 2 Ronald, P. C. & Beutler, B. Plant and animal sensors of conserved microbial signatures.
603 *Science* **330**, 1061-1064, doi:10.1126/science.1189468 (2010).
- 604 3 Dodds, P. N. & Rathjen, J. P. Plant immunity: towards an integrated view of plant-
605 pathogen interactions. *Nat Rev Genet* **11**, 539-548, doi:10.1038/nrg2812 (2010).
- 606 4 Win, J. *et al.* Effector Biology of Plant-Associated Organisms: Concepts and
607 Perspectives. *Cold Spring Harb Symp Quant Biol* **77**, 235-247,
608 doi:doi:10.1101/sqb.2012.77.015933 (2012).
- 609 5 Bialas, A. *et al.* Lessons in Effector and NLR Biology of Plant-Microbe Systems. *Mol*
610 *Plant Microbe Interact* **31**, 34-45, doi:10.1094/MPMI-08-17-0196-FI (2017).
- 611 6 Ellis, J. G., Lawrence, G. J., Luck, J. E. & Dodds, P. N. Identification of regions in alleles
612 of the flax rust resistance gene L that determine differences in gene-for-gene
613 specificity. *Plant Cell* **11**, 495-506 (1999).
- 614 7 Allen, R. L. *et al.* Host-parasite coevolutionary conflict between Arabidopsis and
615 downy mildew. *Science* **306**, 1957-1960, doi:10.1126/science.1104022 (2004).
- 616 8 Bhullar, N. K., Zhang, Z., Wicker, T. & Keller, B. Wheat gene bank accessions as a source
617 of new alleles of the powdery mildew resistance gene Pm3: a large scale allele mining
618 project. *BMC Plant Biol* **10**, 88, doi:10.1186/1471-2229-10-88 (2010).
- 619 9 Seeholzer, S. *et al.* Diversity at the Mla powdery mildew resistance locus from
620 cultivated barley reveals sites of positive selection. *Mol Plant Microbe Interact* **23**,
621 497-509, doi:10.1094/MPMI-23-4-0497 (2010).
- 622 10 Srichumpa, P., Brunner, S., Keller, B. & Yahiaoui, N. Allelic series of four powdery
623 mildew resistance genes at the Pm3 locus in hexaploid bread wheat. *Plant Physiol* **139**,
624 885-895, doi:10.1104/pp.105.062406 (2005).
- 625 11 Lu, X. *et al.* Allelic barley MLA immune receptors recognize sequence-unrelated
626 avirulence effectors of the powdery mildew pathogen. *Proc Natl Acad Sci U S A* **113**,
627 E6486-E6495, doi:10.1073/pnas.1612947113 (2016).
- 628 12 Dodds, P. N. *et al.* Direct protein interaction underlies gene-for-gene specificity and
629 coevolution of the flax resistance genes and flax rust avirulence genes. *Proc Natl Acad*
630 *Sci U S A* **103**, 8888-8893, doi:10.1073/pnas.0602577103 (2006).
- 631 13 Krasileva, K. V., Dahlbeck, D. & Staskawicz, B. J. Activation of an Arabidopsis resistance
632 protein is specified by the in planta association of its leucine-rich repeat domain with
633 the cognate oomycete effector. *Plant Cell* **22**, 2444-2458,
634 doi:10.1105/tpc.110.075358 (2010).
- 635 14 Steinbrenner, A. D., Goritschnig, S. & Staskawicz, B. J. Recognition and activation
636 domains contribute to allele-specific responses of an Arabidopsis NLR receptor to an
637 oomycete effector protein. *PLoS Pathog* **11**, e1004665,
638 doi:10.1371/journal.ppat.1004665 (2015).
- 639 15 Wang, C. I. *et al.* Crystal structures of flax rust avirulence proteins AvrL567-A and -D
640 reveal details of the structural basis for flax disease resistance specificity. *Plant Cell*
641 **19**, 2898-2912, doi:10.1105/tpc.107.053611 (2007).
- 642 16 Huang, J., Si, W., Deng, Q., Li, P. & Yang, S. Rapid evolution of avirulence genes in rice
643 blast fungus *Magnaporthe oryzae*. *BMC Genet* **15**, 45, doi:10.1186/1471-2156-15-45
644 (2014).
- 645 17 Raffaele, S. *et al.* Genome evolution following host jumps in the Irish potato famine
646 pathogen lineage. *Science* **330**, 1540-1543, doi:10.1126/science.1193070 (2010).

- 647 18 Yoshida, K. *et al.* Association genetics reveals three novel avirulence genes from the
648 rice blast fungal pathogen *Magnaporthe oryzae*. *Plant Cell* **21**, 1573-1591,
649 doi:10.1105/tpc.109.066324 (2009).
- 650 19 Dangl, J. L., Horvath, D. M. & Staskawicz, B. J. Pivoting the plant immune system from
651 dissection to deployment. *Science* **341**, 746-751, doi:10.1126/science.1236011
652 (2013).
- 653 20 Rodriguez-Moreno, L., Song, Y. & Thomma, B. P. Transfer and engineering of immune
654 receptors to improve recognition capacities in crops. *Curr Opin Plant Biol* **38**, 42-49,
655 doi:10.1016/j.pbi.2017.04.010 (2017).
- 656 21 Eitas, T. K. & Dangl, J. L. NB-LRR proteins: pairs, pieces, perception, partners, and
657 pathways. *Curr Opin Plant Biol* **13**, 472-477, doi:10.1016/j.pbi.2010.04.007 (2010).
- 658 22 Wu, C. H., Belhaj, K., Bozkurt, T. O., Birk, M. S. & Kamoun, S. Helper NLR proteins
659 NRC2a/b and NRC3 but not NRC1 are required for Pto-mediated cell death and
660 resistance in *Nicotiana benthamiana*. *New Phytol* **209**, 1344-1352,
661 doi:10.1111/nph.13764 (2016).
- 662 23 Narusaka, M. *et al.* RRS1 and RPS4 provide a dual Resistance-gene system against
663 fungal and bacterial pathogens. *Plant J* **60**, 218-226, doi:10.1111/j.1365-
664 313X.2009.03949.x (2009).
- 665 24 Sinapidou, E. *et al.* Two TIR:NB:LRR genes are required to specify resistance to
666 *Peronospora parasitica* isolate Cala2 in *Arabidopsis*. *Plant J* **38**, 898-909,
667 doi:10.1111/j.1365-313X.2004.02099.x (2004).
- 668 25 Ashikawa, I. *et al.* Two adjacent nucleotide-binding site-leucine-rich repeat class genes
669 are required to confer Pikm-specific rice blast resistance. *Genetics* **180**, 2267-2276,
670 doi:10.1534/genetics.108.095034 (2008).
- 671 26 Lee, S. K. *et al.* Rice Pi5-mediated resistance to *Magnaporthe oryzae* requires the
672 presence of two coiled-coil-nucleotide-binding-leucine-rich repeat genes. *Genetics*
673 **181**, 1627-1638, doi:10.1534/genetics.108.099226 (2009).
- 674 27 Wu, C. H. *et al.* NLR network mediates immunity to diverse plant pathogens. *Proc Natl*
675 *Acad Sci U S A* **114**, 8113-8118, doi:10.1073/pnas.1702041114 (2017).
- 676 28 Cesari, S., Bernoux, M., Moncuquet, P., Kroj, T. & Dodds, P. N. A novel conserved
677 mechanism for plant NLR protein pairs: the "integrated decoy" hypothesis. *Front Plant*
678 *Sci* **5**, 606, doi:10.3389/fpls.2014.00606 (2014).
- 679 29 Kroj, T., Chanclud, E., Michel-Romiti, C., Grand, X. & Morel, J. B. Integration of decoy
680 domains derived from protein targets of pathogen effectors into plant immune
681 receptors is widespread. *New Phytol* **210**, 618-626, doi:10.1111/nph.13869 (2016).
- 682 30 Sarris, P. F., Cevik, V., Dagdas, G., Jones, J. D. & Krasileva, K. V. Comparative analysis
683 of plant immune receptor architectures uncovers host proteins likely targeted by
684 pathogens. *BMC Biol* **14**, 8, doi:10.1186/s12915-016-0228-7 (2016).
- 685 31 Wu, C. H., Krasileva, K. V., Banfield, M. J., Terauchi, R. & Kamoun, S. The "sensor
686 domains" of plant NLR proteins: more than decoys? *Front Plant Sci* **6**, 134,
687 doi:10.3389/fpls.2015.00134 (2015).
- 688 32 Okuyama, Y. *et al.* A multifaceted genomics approach allows the isolation of the rice
689 Pia-blast resistance gene consisting of two adjacent NBS-LRR protein genes. *Plant J*
690 **66**, 467-479, doi:10.1111/j.1365-313X.2011.04502.x (2011).
- 691 33 Maqbool, A. *et al.* Structural basis of pathogen recognition by an integrated HMA
692 domain in a plant NLR immune receptor. *Elife* **4**, doi:10.7554/eLife.08709 (2015).
- 693 34 Kanzaki, H. *et al.* Arms race co-evolution of *Magnaporthe oryzae* AVR-Pik and rice Pik
694 genes driven by their physical interactions. *Plant J* **72**, 894-907, doi:10.1111/j.1365-
695 313X.2012.05110.x (2012).

696 35 Costanzo, S. & Jia, Y. L. Sequence variation at the rice blast resistance gene Pi-km
697 locus: Implications for the development of allele specific markers. *Plant Sci* **178**, 523-
698 530, doi:10.1016/j.plantsci.2010.02.014 (2010).

699 36 Krissinel, E. Stock-based detection of protein oligomeric states in jsPISA. *Nucleic Acids*
700 *Res* **43**, W314-319, doi:10.1093/nar/gkv314 (2015).

701 37 de Guillen, K. *et al.* Structure Analysis Uncovers a Highly Diverse but Structurally
702 Conserved Effector Family in Phytopathogenic Fungi. *PLoS Pathog* **11**, e1005228,
703 doi:10.1371/journal.ppat.1005228 (2015).

704 38 Bent, A. F. *et al.* RPS2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant
705 disease resistance genes. *Science* **265**, 1856-1860 (1994).

706 39 Mindrinos, M., Katagiri, F., Yu, G. L. & Ausubel, F. M. The *A. thaliana* disease resistance
707 gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich
708 repeats. *Cell* **78**, 1089-1099 (1994).

709 40 Whitham, S. *et al.* The product of the tobacco mosaic virus resistance gene N:
710 similarity to toll and the interleukin-1 receptor. *Cell* **78**, 1101-1115 (1994).

711 41 Le Roux, C. *et al.* A receptor pair with an integrated decoy converts pathogen disabling
712 of transcription factors to immunity. *Cell* **161**, 1074-1088,
713 doi:10.1016/j.cell.2015.04.025 (2015).

714 42 Sarris, P. F. *et al.* A Plant Immune Receptor Detects Pathogen Effectors that Target
715 WRKY Transcription Factors. *Cell* **161**, 1089-1100, doi:10.1016/j.cell.2015.04.024
716 (2015).

717 43 Zhang, Z. M. *et al.* Mechanism of host substrate acetylation by a YopJ family effector.
718 *Nat Plants* **3**, 17115, doi:10.1038/nplants.2017.115 (2017).

719 44 Ortiz, D. *et al.* Recognition of the *Magnaporthe oryzae* Effector AVR-Pia by the Decoy
720 Domain of the Rice NLR Immune Receptor RGA5. *Plant Cell* **29**, 156-168,
721 doi:10.1105/tpc.16.00435 (2017).

722 45 Lobstein, J. *et al.* SHuffle, a novel *Escherichia coli* protein expression strain capable of
723 correctly folding disulfide bonded proteins in its cytoplasm. *Microb Cell Fact* **11**, 56,
724 doi:10.1186/1475-2859-11-56 (2012).

725 46 Studier, F. W. Protein production by auto-induction in high density shaking cultures.
726 *Protein Expr Purif* **41**, 207-234 (2005).

727 47 Wickham, H. *ggplot. Elegant Graphics for Data Analysis.* (Springer-Verlag New York,
728 2009).

729 48 Winter, G. xia2: an expert system for macromolecular crystallography data reduction.
730 *Journal of Applied Crystallography* **43**, 186-190, doi:10.1107/S0021889809045701
731 (2010).

732 49 Evans, P. R. & Murshudov, G. N. How good are my data and what is the resolution?
733 *Acta Crystallogr D* **69**, 1204-1214, doi:10.1107/S0907444913000061 (2013).

734 50 Winn, M. D. *et al.* Overview of the CCP4 suite and current developments. *Acta*
735 *Crystallogr D Biol Crystallogr* **67**, 235-242, doi:10.1107/S0907444910045749 (2011).

736 51 McCoy, A. J. *et al.* Phaser crystallographic software. *J Appl Crystallogr* **40**, 658-674,
737 doi:10.1107/S0021889807021206 (2007).

738 52 Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot.
739 *Acta Crystallogr D Biol Crystallogr* **66**, 486-501, doi:10.1107/S0907444910007493
740 (2010).

741 53 Murshudov, G. N. *et al.* REFMAC5 for the refinement of macromolecular crystal
742 structures. *Acta Crystallogr D Biol Crystallogr* **67**, 355-367,
743 doi:10.1107/S0907444911001314 (2011).

744 54 Chen, V. B. *et al.* MolProbity: all-atom structure validation for macromolecular
745 crystallography. *Acta Crystallogr D Biol Crystallogr* **66**, 12-21,
746 doi:10.1107/S0907444909042073 (2010).

