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Polymorphic residues in rice NLRs expand binding and response to effectors of the blast pathogen

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Supplementary Data

Supplementary Methods

Gene cloning

For protein expression aimed at in vitro studies, DNA encoding the Pikp-HMA domain (amino acid residues Gly186-Asp263) or Pikm-HMA domain (residues Gly186-Asp264) were amplified using standard PCR reactions, and appropriate templates, followed by cloning into the pOPINM vector using standard protocols[1]. AVR-Pik expression constructs used are as described in[2], with the exception of the AVR-Pik(Arg53Glu) mutants, which were generated using standard molecular biology techniques and subcloned into pOPINE with an N-terminal cleavable MBP tag.

For Y2H, Pik-HMA domains and AVR-Pik effectors/mutants were cloned into pGBKT7 and pGADT7, respectively. Plasmids were linearized by double digestion with EcoRI and BamHI (New England Biolabs) and genes of interest were introduced using the In-Fusion cloning kit (Takara Bio USA).

For in planta studies, full-length Pik-1 and Pik-2 NLRs, and all AVR-Pik variants/mutants, were assembled using Golden Gate methods[3]. Sequences were domesticated to remove BsaI and BbsI restriction sites. Pik-1 NLRs were cloned into pICH47742 with C-terminal 6xHis/3xFlag tag, Pik-2 NLRs were cloned into pICH47751 with C-terminal 6xHA tag. All NLRs were expressed driven by the *A. tumefaciens* Mas promoter and terminator. AVR-Pik effectors/mutants were cloned into pICH47751 with an N-terminal 4xMyc tag, and expression driven by the *A. thaliana* Ubi10 promoter with a 35S terminator.

All DNA constructs were verified by sequencing.

Purification of proteins for in vitro binding studies

Cells were harvested by centrifugation and re-suspended in 50 mM Tris-HCl pH7.5, 500 mM NaCl, 50 mM Glycine, 5% (vol/vol) glycerol and 20 mM imidazole (buffer A) supplemented with EDTA-free protease inhibitor tablets (Roche). The cells were lysed by sonication and the clarified lysate was applied to a Ni^{2+}-NTA column connected to an AKTA Xpress purification system (GE Healthcare). Proteins were step-eluted with elution buffer (buffer A containing 500 mM imidazole) and directly injected onto a Superdex 75 26/60 gel filtration column pre-equilibrated with 20 mM HEPES pH7.5, 150 mM NaCl (buffer B). Purification tags were removed by incubation with 3C protease (10 µg/mg fusion protein) followed by passing through tandem Ni^{2+}-NTA and MBP Trap HP columns (GE Healthcare). The flow-through was concentrated as appropriate and loaded on a Superdex 75 26/60 gel filtration column for final purification and buffer exchange into buffer B.

SPR cycling conditions

The system was maintained at 25 °C, and a flow rate of 30 µl/min was used. For each

kinetic cycle, 30 µl of 0.5 mM $NiCl_2$ was used to activate the chip and, after washing, His-tagged AVR-Pik variants were immobilised on the sample cell, giving a response level of 240 ± 50. Concentrations of Pik-HMA (ranging from 0 - 200 nM for PikmHMA, and 0 – 100 nM for Pikp-HMA) were injected over both reference and sample cells for 360s, and dissociation was recorded for further 120s. The sensor chip was regenerated between each cycle with 30 µl of 0.35M EDTA. For each measurement, in addition to subtracting the response in the reference cell, a further buffer-only subtraction was made to correct for bulk refractive index changes or machine effects[4].

Complexes crystallization conditions

Each complex crystallised as follows:

(i) Pikm-HMA/AVR-PikD (12.5mg/ml), Morpheus® HT-96 condition E10 [0.12M Ethylene glycols (0.3M Diethylene glycol; 0.3M Triethylene-glycol; 0.3M Tetraethylene glycol; 0.3M Pentaethylene glycol); 0.1M Buffer system 3 (1M Tris (base); BICINE) pH 8.5; 50% v/v Precipitant mix 2 (40% v/v Ethylene glycol; 20 % w/v PEG 8000)];

(ii) Pikm-HMA/AVR-PikE (6.2 mg/ml), Morpheus® HT-96 condition H6 [0.1M Amino acids (0.2M L-Na-Glutamate; 0.2M Alanine (racemic); 0.2M Glycine; 0.2M Lysine HCl (racemic); 0.2M Serine (racemic)); 0.1M Buffer system 2 (1M sodium HEPES, MOPS (acid)) pH 7.5; 50% v/v Precipitant mix 2 (40% v/v Ethylene glycol; 20 % w/v PEG 8000)];

(iii) Pikm-HMA/AVR-PikA (9.5 mg/ml), Morpheus® HT-96 condition D18 [0.12M Alcohols (0.2M 1,6-Hexanediol; 0.2M 1-Butanol; 0.2M 1,2-Propanediol; 0.2M 2-Propanol; 0.2M 1,4-Butanediol; 0.2M 1,3-Propanediol); 0.1M Buffer system 2 (1M sodium HEPES, MOPS (acid)) pH 7.5; 50% v/v Precipitant mix 4 (25%v/v MPD; 25%v/v PEG 1000; 25%v/v PEG3350)];

(iv) Pikp-HMA/AVR-PikD (15 mg/ml), Morpheus® HT-96 condition D4 [0.12M Alcohols (0.2M 1,6-Hexanediol; 0.2M 1-Butanol; 0.2M 1,2-Propanediol; 0.2M 2-Propanol; 0.2M 1,4-Butanediol; 0.2M 1,3-Propanediol); 0.1M Buffer system 1 (1M Imidazole; MES monohydrate (acid)) pH 6.5; 50% v/v Precipitant mix 4 (25%v/v MPD; 25%v/v PEG 1000; 25%v/v PEG3350)];

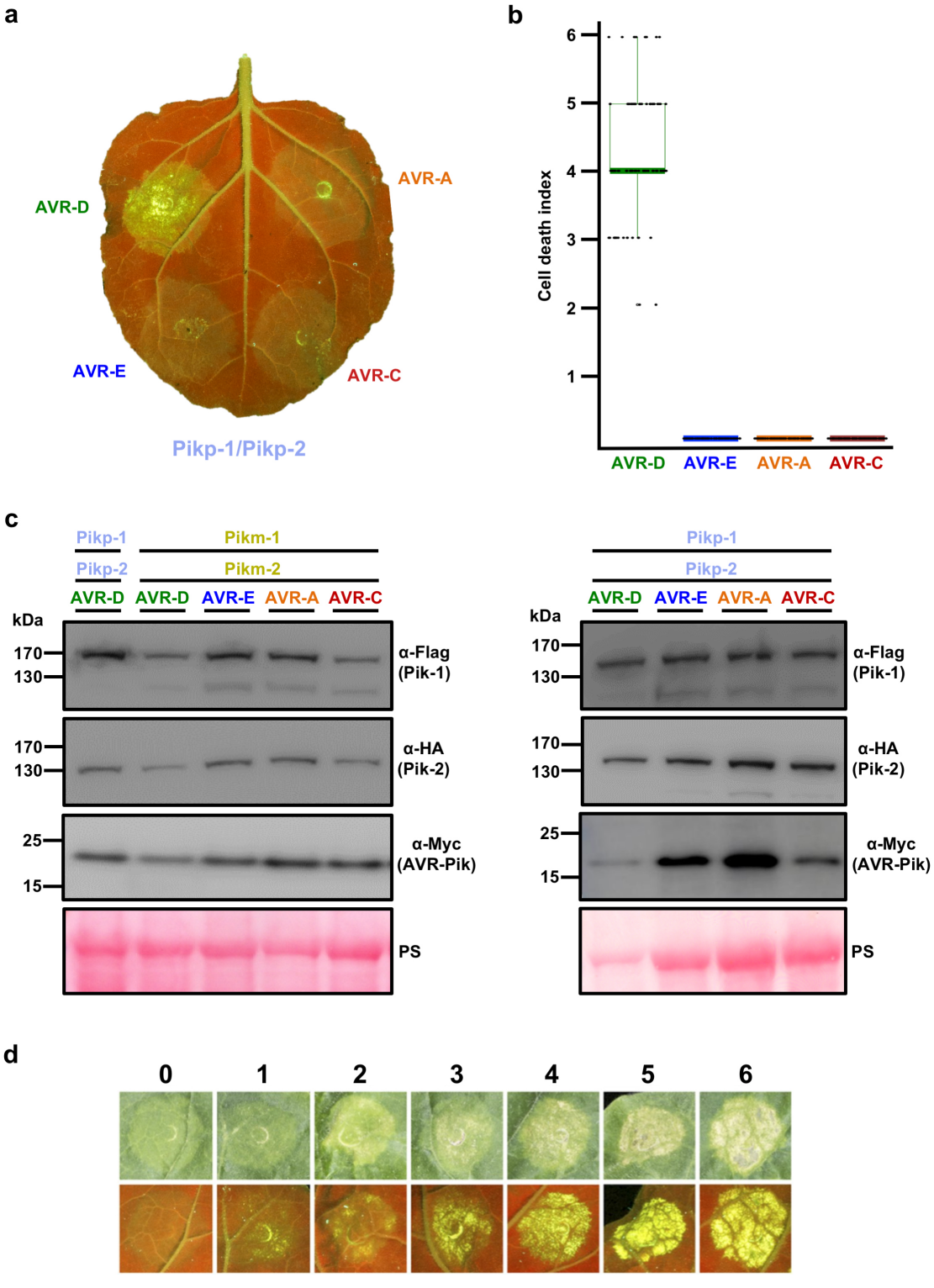
(v) Pikp-HMA/AVR-PikE (28.4 mg/ml), Morpheus® HT-96 condition A4 [0.06M Divalents (0.3M Magnesium chloride hexahydrate; 0.3M Calcium chloride dihydrate); 0.1M Buffer system 1 (1M Imidazole; MES monohydrate (acid)) pH 6.5; 50% v/v Precipitant mix 4 (25%v/v MPD; 25%v/v PEG 1000; 25%v/v PEG3350)].

Additional data collection and refinement information

All data sets were collected at 100 °K.

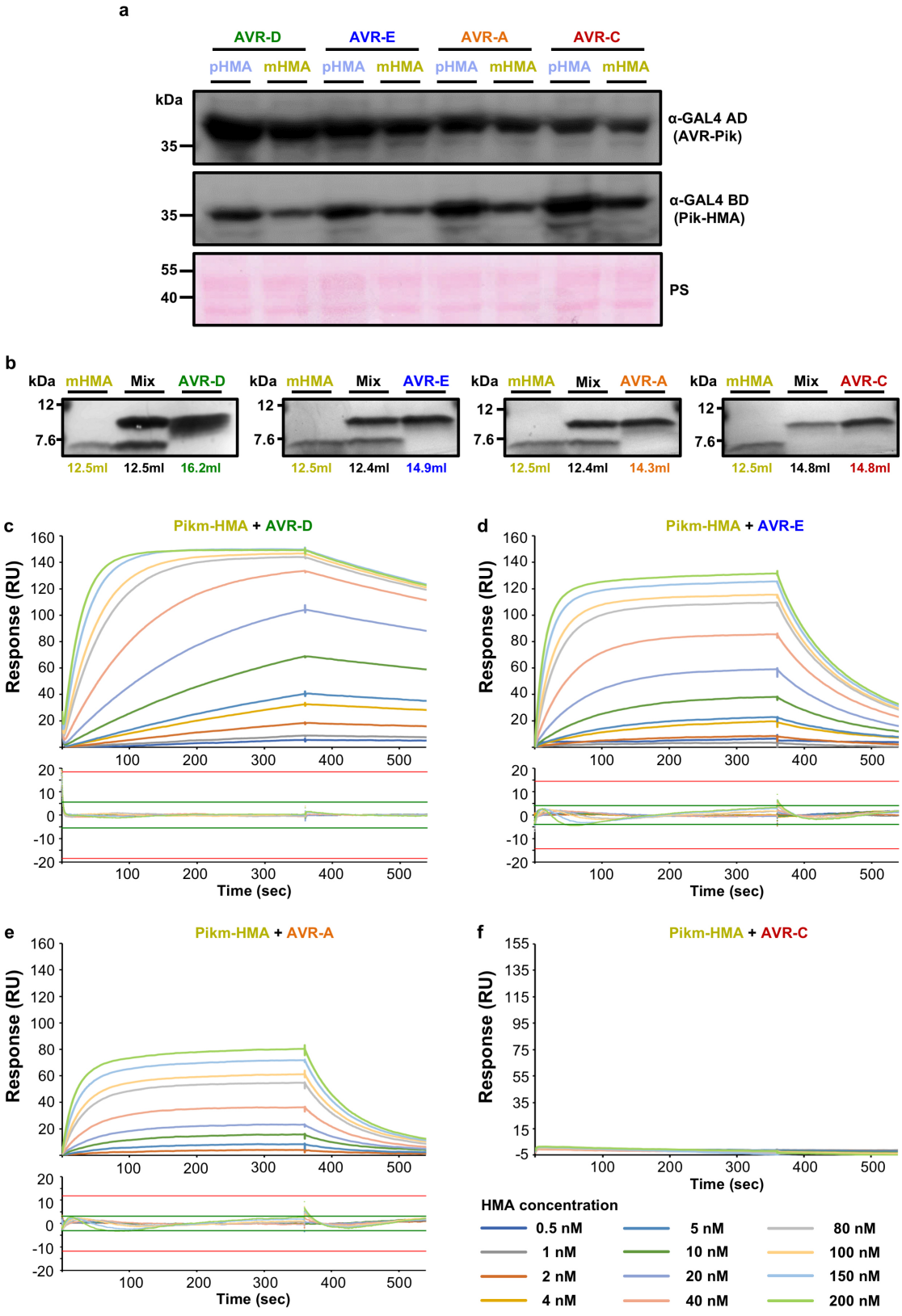
	Pikm/AVR-PikD	Pikm/AVR-PikE	Pikm/AVR-PikA	Pikp/AVR-PikD	Pikp/AVR-PikE
Data collection					
Beamline	I03	I04	I04	I03	I03
Wavelength (Å)	0.9168	0.9795	0.9795	0.9763	0.9763
CC(1/2) (%)#	99.9 (64.4)	99.9 (73.9)	99.9 (94.1)	99.8 (82.6)	99.9 (94.7)
Refinement					
Ramachandran plot (%)**					
Favoured	99.1	98.2	99.4	98.2	98.8
Allowed	0.9	1.8	0.6	1.8	1.2
Outliers	0	0	0	0	0
MolProbity Score	0.92 (99th percentile)	0.50 (100th percentile)	0.83 (100th percentile)	1.36 (90th percentile)	1.23 (99th percentile)

#As calculated by xia2 pipeline or Aimless, **As calculated by MolProbity



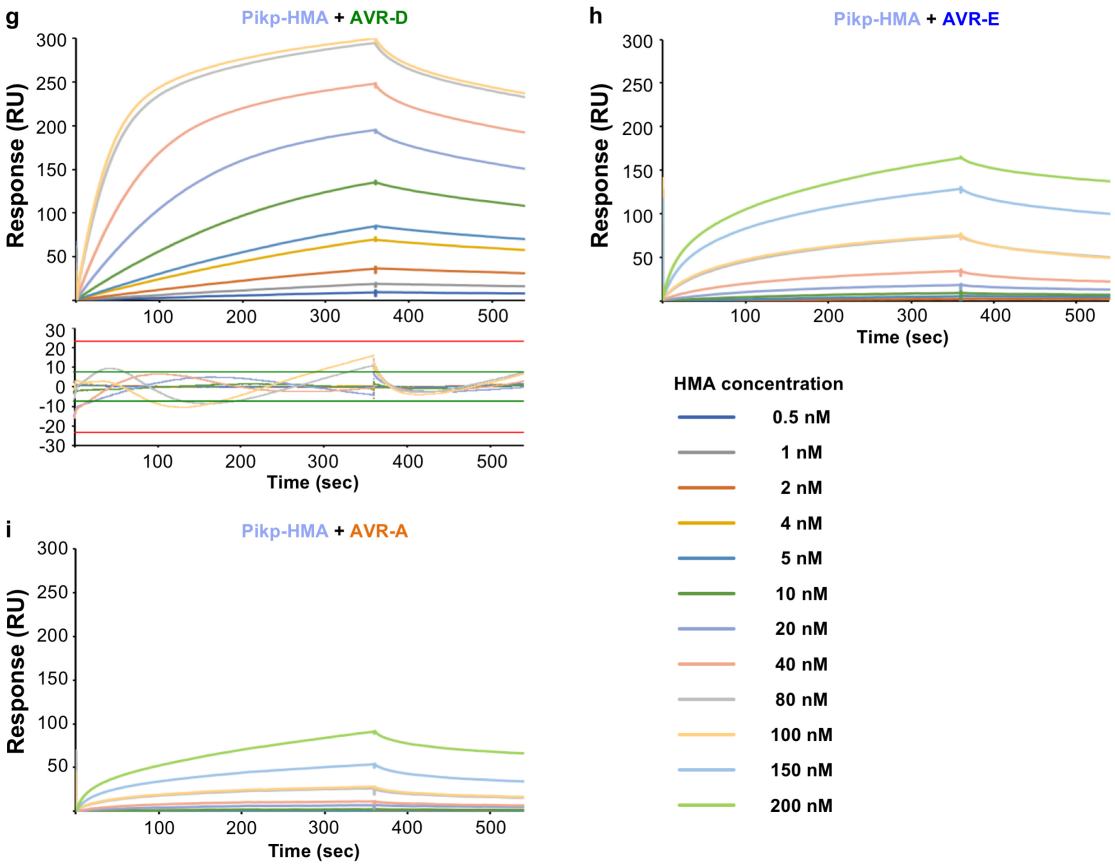
Supplementary Figure 1: (a) Representative leaf images depicting Pikp-mediated cell death as autofluorescence under UV-light using the same expression vectors as for Pikm, **(b)** Box-plots showing repeats of the cell death assay for Pikp-mediated cell death, for each sample the number of repeats was 80. The centre line represents the median, box limits are upper and lower quartiles, whiskers are 1.5x interquartile range, and all data points are represented as dots.

(c) Western blots showing expression of AVR-Pik variants and Pik NLRs (Pik-1 proteins are detected as a double band by the FLAG antibody in our blots), PS = Ponceau Stain. Each experiment was repeated a minimum of 3 times, with similar results. **(d)** Cell death scoring criteria, as previously published[2]. For brevity, effectors are labelled without the 'Pik' designation where appropriate.

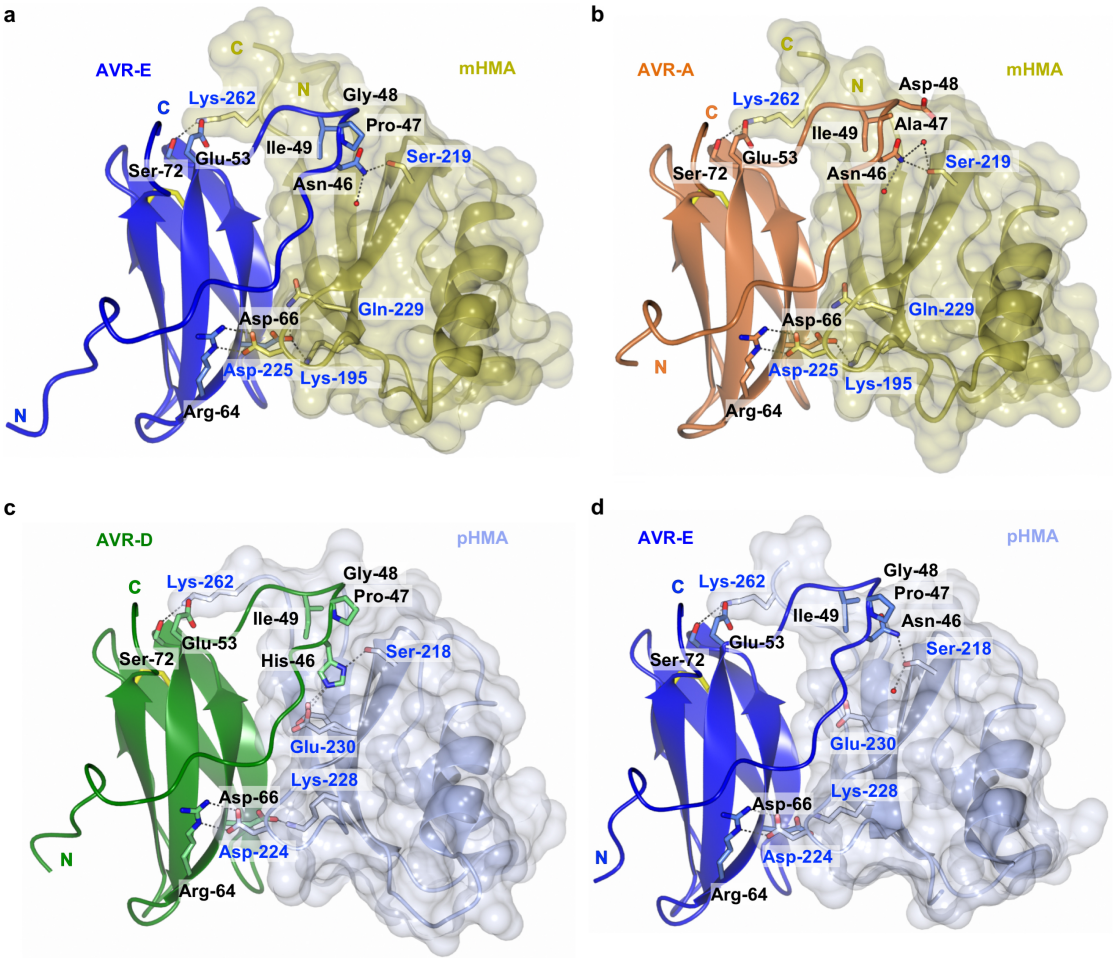


Supplementary Figure 2(a-f): (a) Western blot analysis confirming expression of proteins in yeast (AD = activation domain, BD = binding domain), PS = Ponceau Stain, **(b)** SDS-PAGE showing the proteins eluted under the peaks in the analytical gel

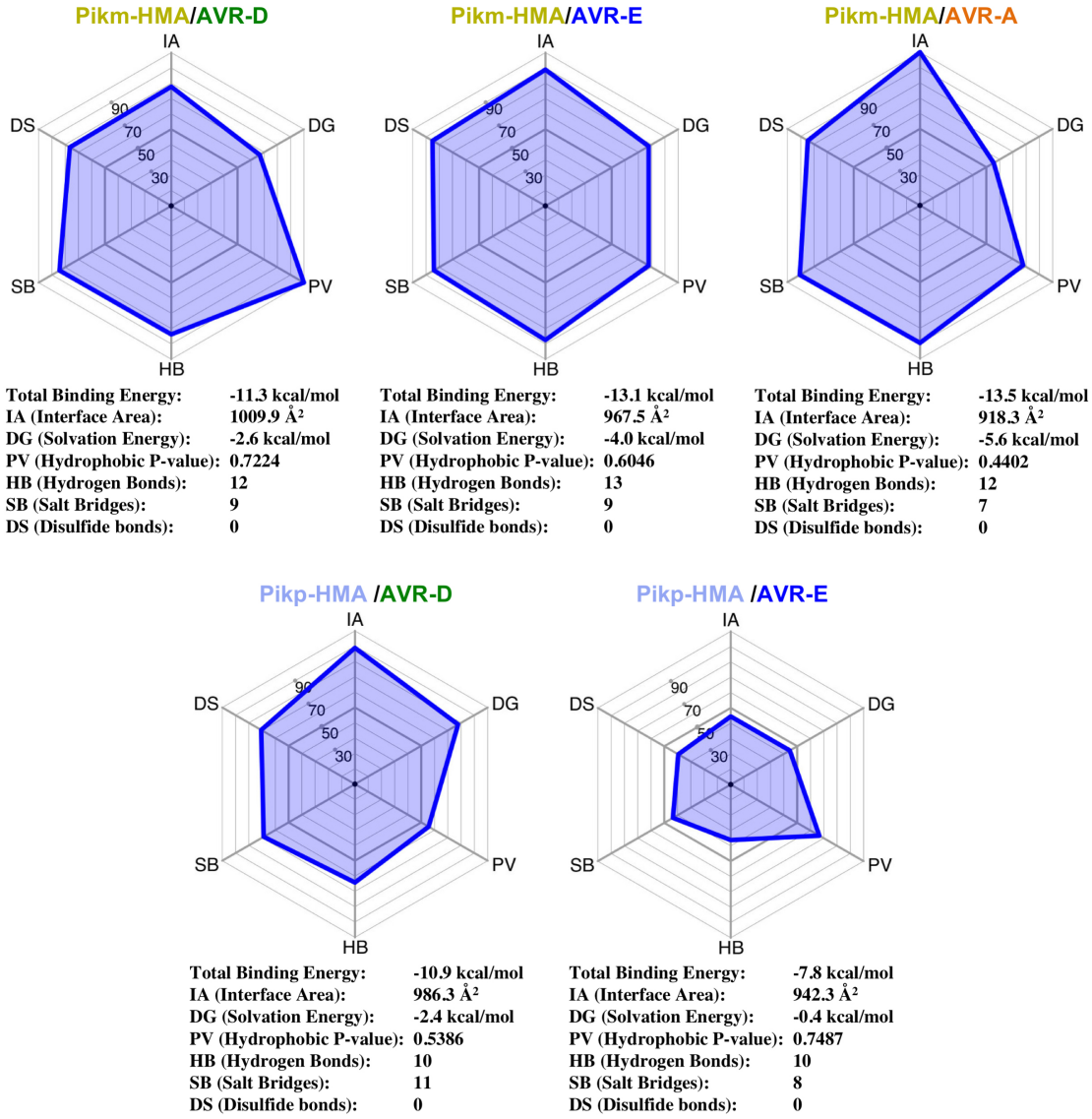
filtration experiments shown in **Fig. 2b**. (**c-f)** Multi-cycle kinetics data for the interaction of Pikm-HMA (analyte) with effectors **(c)** AVR-PikD, **(d)** AVR-PikE, **(e)** AVR-PikA, **(f)** AVR-PikC. Response units for each labelled protein concentration are shown with the residuals plot beneath (SPR acceptance guides as determined by Biacore software are shown as green and red lines in the residuals plots). These data have been used to calculate the association/dissociation constants given in **Supplementary Table 1**. Each experiment represented in panels **a-f** was repeated a minimum of 3 times, with similar results.



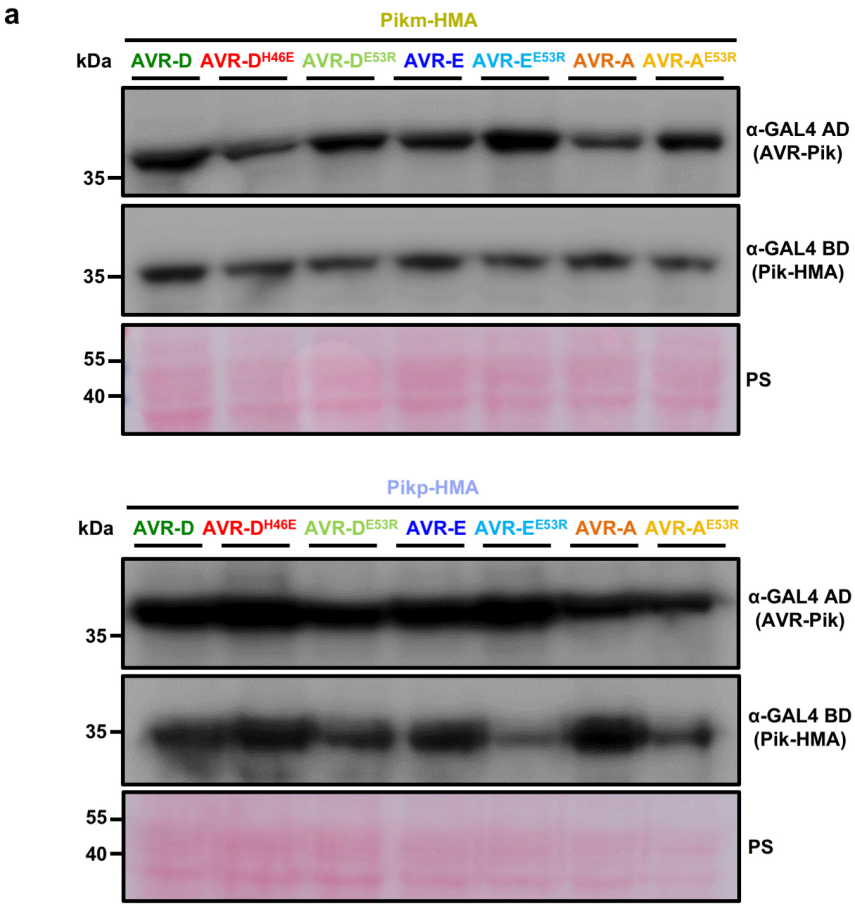
Supplementary Figure 2: (g-i) Multi-cycle kinetics data for the interaction of Pikp-HMA (analyte) with effectors **(g)** AVR-PikD, **(h)** AVR-PikE, **(i)** AVR-PikA. Response units for each labelled protein concentration are shown with the residuals plot beneath. The data in panel **(g)** was used to calculate the association/dissociation constants given in **Supplementary Table 1** (SPR acceptance guides as determined by Biacore software are shown as green and red lines in the residuals plots). Each experiment represented in panels **g-i** was repeated a minimum of 3 times, with similar results.



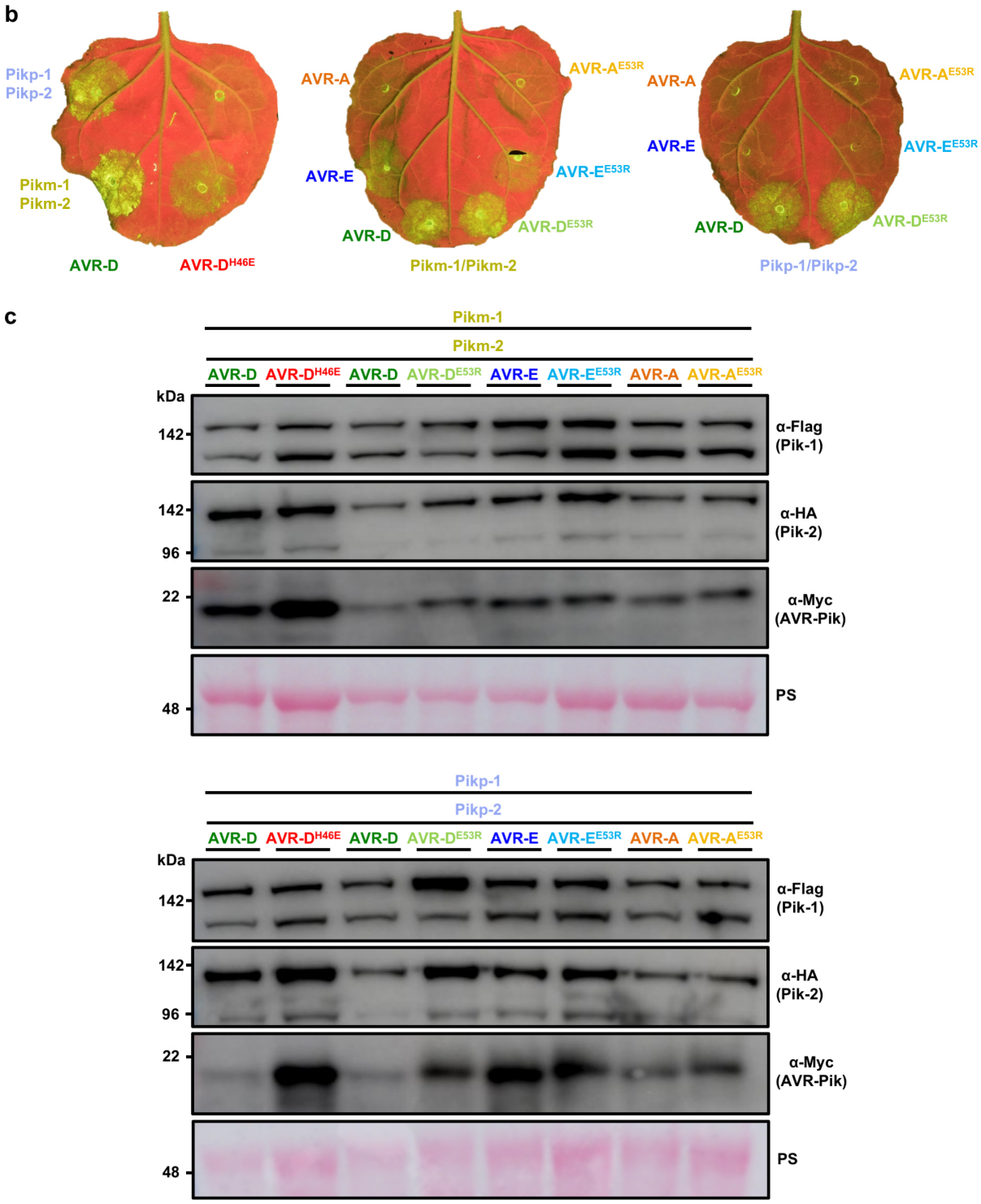
Supplementary Figure 3: Structures of Pikm-HMA and Pikp-HMA in complex with AVR-Pik effectors. Schematic representations of the structures of Pikm-HMA in complex with **(a)** AVR-PikE and **(b)** AVR-PikA, and Pikp-HMA in complex with **(c)** AVR-PikD and **(d)** AVR-PikE. The Pikm-HMA/AVR-Pik complexes are shown as detailed in **Fig. 3a**, but with the effectors in blue and orange respectively. In panels **(c)** and **(d)**, Pikp-HMA is shown in ice blue cartoon representation with selected side chains as sticks; the molecular surface of this domain is also shown. The effectors are coloured as labelled, and shown in cartoon representation, with selected side chains as sticks. Hydrogen bonds/salt bridges are shown as dashed lines and the di-sulfide bond as yellow bars. For clarity, of the two molecules of Pikp-HMA present in the complex, only the one making extensive contacts with the effector is shown.



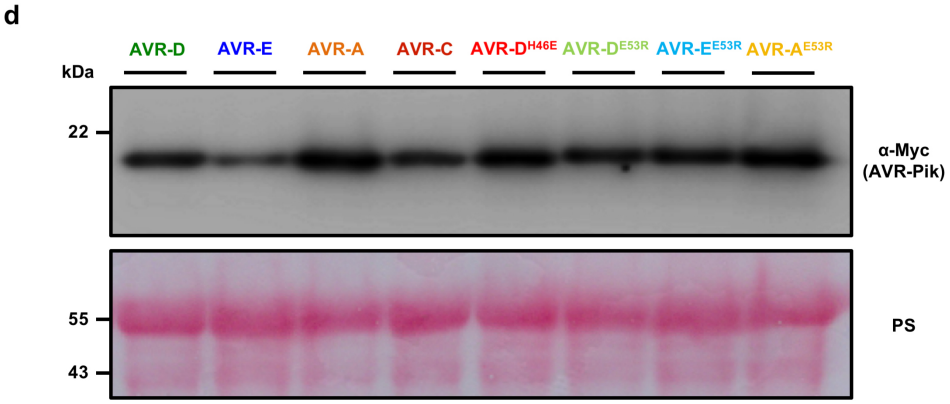
Supplementary Figure 4: Analyses of Pikm-HMA and Pikp-HMA interfaces with AVR-Pik effectors using QtPISA. For each complex (as labelled), the QtPISA interaction radar generated with the reference parameter "Total Binding Energy" is shown. In this presentation the internal area of the polygon represents the Total Binding Energy. Also shown are the values obtained for each key interface parameter in the analysis. The QtPISA interaction radars score macromolecular interfaces based on statistical analysis of all interfaces found in the Protein DataBank (see **Methods**). The abbreviations in the radars are defined in the tables underneath each panel.



Supplementary Figure 5: (a) Western blot analysis confirming expression of proteins in yeast (AD = activation domain, BD = binding domain), PS = Ponceau staining. Each experiment was repeated a minimum of 3 times, with similar results.



Supplementary Figure 5: (b) Side-by-side leaf images showing cell death in *N. benthamiana* for the effector mutants at position 46 and 53, with Pikm and Pikp. Leaves are representative of 3 independent experiments, comprising a total of 90 replicas, quantified in Fig. 6c. **(c)** Western blots showing the expression of each protein in leaf tissue. Each experiment was repeated a minimum of 3 times, with similar results.



Supplementary Figure 5: (d) Western blots showing the accumulation of effector variants and mutants in *N. benthamiana* in the absence of Pik NLRs. This experiment was repeated a minimum of 3 times, with similar results.

Supplementary Table 1: Association and dissociation constants for Pik-HMA and effector variants from the multi-cycle kinetics SPR data

Interaction	$k_a \pm$ SE ($10^5 M^{-1} s^{-1}$)	$k_d \pm$ SE ($10^{-3} s^{-1}$)	K_D
Pikm/AVR-PikD	2.195 ± 0.004	1.027 ± 0.002	4.7 nM
Pikm/AVR-PikE	2.986 ± 0.010	8.60 ± 0.05	29 nM
Pikm/AVR-PikA	1.530 ± 0.009	11.16 ± 0.05	73 nM
Pikp/AVR-PikD	2.077 ± 0.002	1.217 ± 0.003	5.9 nM

Supplementary Table 2: X-ray Data Collection and Refinement Table

	Pikm/AVR-PikD	Pikm/AVR-PikE	Pikm/AVR-PikA	Pikp/AVR-PikD	Pikp/AVR-PikE
Data collection					
Space group	$P2_12_12_1$	$P2_1$	$P2_1$	$P2_1$	$P2_12_12_1$
Cell dimensions					
a, *b*, *c* (Å)	37.09, 87.13, 103.84	30.02, 54.35, 48.49	30.07, 54.44, 46.64	40.50, 66.26, 45.74	67.20, 80.18, 105.63
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.53, 90.00	90.00, 104.43, 90.00	90.00, 113.32, 90.00	90.00, 90.00, 90.00
Resolution (Å)*	29.04-1.20 (1.23-1.20)	23.70-1.30 (1.32-1.30)	34.76-1.30 (1.33-1.30)	42.00-1.35 (1.37-1.35)	46.29-1.90 (1.94-1.90)
R_{merge}(%); R_{meas} (%)#	4.9 (85.8); 5.2 (97.2)	7.1 (90.4); 7.6 (101.0)	4.3 (28.4); 4.8 (35.2)	6.1 (76.4); 6.6 (83.1)	10.0 (100.5); 10.4 (105)
$(I)/\sigma(I)$#	21.7 (2.3)	12.9 (1.6)	21.5 (4.5)	13.4 (2.4)	14.5 (2.6)
Completeness (%)#	98.5 (88.4)	99.4 (92.4)	99.3 (91.9)	99.8 (99.8)	100.0 (99.9)
Unique reflections#	104301 (6772)	38170 (1751)	33854 (2405)	48646 (2371)	45720 (2876)
Redundancy#	11.9 (7.9)	7.6 (5.0)	7.8 (5.2)	6.6 (6.6)	12.0 (12.1)
Refinement					
Resolution (Å)	29.04-1.20 (1.23-1.20)	23.70-1.30 (1.33-1.30)	34.76-1.30 (1.33-1.30)	42.00-1.35 (1.39-1.35)	46.29-1.90 (1.95-1.90)
No. reflections	99199 (6450)	36190 (2514)	33854 (2277)	46253 (3399)	43475 (3145)
R_{work}/R_{free} (%)^	14.9/18.4 (25.8/28.1)	14.1/17.9 (23.9/26.9)	12.5/16.2 (17.1/21.8)	14.3/18.5 (22.7/23.3)	20.9/22.4 (31.9/31.4)
No. atoms					
Protein	2628	1384	1292	1823	3482
Ligand/ion	-	-	-	1	
Water	291	144	144	200	230
B-factors					
Protein	22.5	21.0	21.9	22.8	36.1
Ligand/ion				51.5	
Water	32.6	33.2	34.8	36.3	38.1
R.m.s. deviations^					
Bond lengths (Å)	0.019	0.018	0.017	0.018	0.014
Bond angles (°)	1.902	1.925	1.775	1.919	1.614

*The highest resolution shell is shown in parenthesis. #As calculated by xia2 pipeline or Aimless, ^As calculated by Refmac5

Supplementary Table 3: Summary of overlays analysis (calculated with SSM in COOT)

Complex (compared to Pikm/AVR-PikD)	AVR-Pik	HMA
	r.m.s.d. - Å (no. of residues)	r.m.s.d. - Å (no. of residues)
Pikm/AVR-PikE	0.55 (82 of 84)	0.54 (78 of 79)
Pikm/AVR-PikA	0.37 (81 of 84)	0.50 (76 of 79)
Pikp/AVR-PikD	0.50 (81 of 84)	1.10 (72 of 79)
Pikp/AVR-PikE	0.48 (82 of 84)	0.99 (72 of 79)

Complex (compared to Pikp/AVR-PikD)	AVR-Pik	HMA
	r.m.s.d. - Å (no. of residues)	r.m.s.d. - Å (no. of residues)
Pikp/AVR-PikE	0.35 (81 of 83)	0.60 (70 of 76)

Supplementary Table 4: Summary of QtPISA analysis

Complex	AVR-Pik		HMA		Total interface area*	No. of H-bonds	No. of Salt Bridges
	B.S.A. (Å^2)	% B.S.A. of total	B.S.A. (Å^2)	% B.S.A. of total	Å^2		
Pikm/AVR-PikD (chains A and B)	1004.9	18.1	1015.0	20.9	1009.9	12	9
Pikm/AVR-PikE	996.2	20.0	939.0	16.3	967.5	13	9
Pikm/AVR-PikA	891.2	16.6	945.4	19.8	918.3	12	7
Pikp/AVR-PikD (chains B and C)	978.4	17.8	994.2	21.2	986.3	10	11
Pikp/AVR-PikE (chains E and F)	969.1	21.3	915.5	17.2	942.3	10	8

*Total interface area is the sum of the B.S.A. (Buried Surface Area) of each component divided by two.

Supplementary References

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