Protein engineering expands the effector recognition profile of a rice NLR immune receptor

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Abstract

Plants NLR receptors detect pathogen effectors and initiate an immune response. Since their discovery, NLRs have been the focus of protein engineering to improve disease resistance. However, this has proven challenging, in part due to their narrow response specificity. Previously, we revealed the structural basis of pathogen recognition by the integrated HMA of the rice NLR Pikp (Maqbool, Saitoh et al. 2015). Here, we used structure-guided engineering to expand the response profile of Pikp to variants of the rice blast pathogen effector AVR-Pik. A mutation located within an effector binding interface of the integrated Pikp-HMA domain increased the binding affinity for AVR-Pik variants in vitro and in vivo. This translates to an expanded cell death response to AVR-Pik variants previously unrecognized by Pikp in planta. Structures of the engineered Pikp-HMA in complex with AVR-Pik variants revealed the mechanism of expanded recognition. These results provide a proof-of-concept that protein engineering can improve the utility of plant NLR receptors where direct interaction between effectors and NLRs is established, particularly via integrated domains.
**Introduction**

Protein engineering offers opportunities to develop new or improved molecular recognition capabilities that have applications in basic research, health and agricultural settings. Protein resurfacing, where the properties of solvent-exposed regions are changed (often by mutation), has been used extensively in diverse areas from antibody engineering for clinical use, to production of more stable, soluble proteins for biotechnology applications (1).

Intracellular nucleotide binding, leucine rich repeat (NLR) receptors are key components of plant innate immunity pathways. They recognise the presence or activity of virulence-associated, host-translocated pathogen effector proteins and initiate an immune response (2,3). As they confer resistance to disease, plant NLRs are widely used in crop breeding programs (4). However, the recognition spectrum of plant NLRs tends to be very specific, and pathogens may delete detected effectors from their genome or evolve novel effector variants not detected by NLRs to re-establish disease (5).

The potential of engineering NLRs to overcome these limitations, or to detect new effector activities, is emerging (6). Some success has been achieved in expanding the activation sensitivity or effector recognition profiles of NLRs through gain-of-function random mutagenesis (7-9). In an alternative strategy, NLR perception of protease effectors, through their activity on engineered host proteins, can lead to expanded recognition profiles (10-12). When detailed knowledge of direct binding interfaces between an effector and an NLR are known, this offers the potential for protein resurfacing to modify interactions, and impact immune signalling.

Plant NLRs are modular proteins, defined by their nucleotide-binding (NB-ARC) and leucine rich repeat (LRR) domains, but also have either an N-terminal coiled-coil (CC) or Toll/Interleukin-1/Resistance-protein (TIR) signalling domain (13). However, many NLRs also contain non-canonical integrated domains (14-16). Integrated domains are thought to be derived from ancestral virulence-associated effector targets which directly bind pathogen effectors (or host proteins (17)), or are
modified by them, to initiate an immune response (18-22). As such, these domains present an exciting target for protein engineering approaches to improve NLR activities. NLRs containing integrated domains (often called the “sensor”) typically function in pairs, requiring a second genetically linked NLR (the “helper”) for immune signalling (23,24).

Two rice NLR pairs, Pik and Pia, contain an integrated Heavy Metal Associated (HMA) domain in their sensor NLR that directly binds effectors from the rice blast pathogen Magnaporthe oryzae (also known as Pycicularia oryzae) (20,25-27). The integrated HMA domain in the sensor NLR Pik-1 directly binds the effector AVR-Pik. Co-evolutionary dynamics has driven the emergence of polymorphic Pik-1 HMA domains and AVR-Pik effectors in natural populations, and these display differential disease resistance phenotypes (28-30). The Pikp NLR allele only responds to the effector variant AVR-PikD, but the Pikm allele responds to AVR-PikD, AVR-PikE, and AVR-PikA. These phenotypes can be recapitulated in the model plant Nicotiana benthamiana using a cell death assay, and are underpinned by differences in effector/receptor binding interfaces that lead to different affinities in vitro (20,22).

We hypothesised that by combining naturally occurring favourable interactions observed across different interfaces, as defined in different Pik-HMA/AVR-Pik structures (20,22), we could generate a Pik NLR with improved recognition profiles. Here, we graft an interface from Pikm onto Pikp by mutating two residues in Pikp (Asn261Lys, Lys262Glu), forming Pikp^{NK-KE}. This single-site mutation strengthens the cell death response in N. benthamiana to AVR-PikD, and gains a Pikm-like response to AVR-PikE and AVR-PikA. We show that this gain-of-function phenotype correlates with increased binding affinity of the effectors by the Pikp^{NK-KE}-HMA domain in vitro and in vivo, and demonstrate this mutation results in a Pikm-like structure for Pikp^{NK-KE}, when in complex with AVR-Pik effectors. Finally, we confirm that the newly engineered interface is responsible for the expanded response of Pikp^{NK-KE} by mutation of the effectors.
This study serves as a proof-of-concept for the use of protein resurfacing by targeted mutation to develop plant NLR immune receptors with new capabilities. In the future, such approaches have the potential to improve disease resistance in crops.
Results

Structure-informed engineering expands Pikp-mediated effector recognition in N. benthamiana

By comparing protein interfaces in the structures of Pikp-HMA and Pikm-HMA bound to different AVR-Pik effectors (20,22), we hypothesised that we could engineer expanded effector recognition capabilities to Pikp by point mutation. We constructed a series of mutations in the previously identified interface 2 and interface 3 regions of Pik-HMA/AVR-Pik structures (22), swapping residues found in Pikm into Pikp (Figure 1A, Figure 1 - figure supplement 1). We then screened these mutations for expanded effector recognition by monitoring cell death in a well-established N. benthamiana assay (20,22). We found one double mutation in two adjacent amino acid residues contained within interface 3, Asn261Lys and Lys262Glu (henceforth PikpNK-KE), showed cell death in response to AVR-PikE and AVR-PikA (Figure 1, Figure 1 - figure supplement 1A,B). Mutations that lose response to AVR-PikD are most likely compromised in effector binding. All proteins were confirmed to be expressed in plants by western blot (Figure 1 - figure supplement 1C).

We subsequently focussed on this mutant, and independently repeated the cell death assay to ensure its robustness (Figure 1B,C, Figure 1 - figure supplement 3). Similar to the Pikm allele (22), we observe a hierarchy of AVR-PikD>AVR-PikE>AVR-PikA for the intensity of cell death mediated by PikpNK-KE. PikpNK-KE shows a comparable, but elevated (not statistically significant in the case of AVR-PikE), response to effector variants compared to Pikm (Figure 1 - figure supplement 2,4). A comparable, but elevated, response is also observed between the cell death of PikpNK-KE with Pikp in response to AVR-PikD (Figure 1B,C). PikpNK-KE does not show a response to the stealthy AVR-PikC variant. To obtain a quantitative measure of cell death, we performed ion leakage assays (Figure 1D, Figure 1 - figure supplement 2, Supplementary File 1). The results of these assays correlate well with the cell death index scoring, based on autofluorescence, described above.
We conclude that the single Asn261Lys/Lys262Glu (Pikp\textsuperscript{NK-KE}) mutation at interface 3 in the Pikp NLR expands this protein’s recognition profile towards effector variants AVR-PikE and AVR-PikA, similar to that observed for Pikm.

The engineered Pikp\textsuperscript{NK-KE}-HMA mutant shows increased binding to effector variants in vivo and in vitro

We used Yeast-2-hybrid (Y2H) and surface plasmon resonance (SPR) to determine whether the expanded Pikp\textsuperscript{NK-KE} cell death response in \textit{N. benthamiana} correlates with increased binding affinity of the Pikp\textsuperscript{NK-KE}-HMA domain for AVR-Pik effectors.

As AVR-PikE and AVR-PikA show some interaction with Pikp-HMA using these approaches (20,22), we tested interactions with Pikp\textsuperscript{NK-KE}-HMA side-by-side with wild-type. By Y2H we observed an increase in growth/blue colouration (both indicative of protein-protein interaction) for Pikp-HMA\textsuperscript{NK-KE} with effectors AVR-PikE and, particularly, AVR-PikA when compared with Pikp-HMA (Figure 2A). This is accentuated with more stringent conditions (imposed by increasing concentration of Aureobasidin A). Unexpectedly, we also observed limited yeast growth for Pikp-HMA\textsuperscript{NK-KE} with AVR-PikC at the lower stringency (Figure 2A). The unrelated \textit{M. oryzae} effector AVR-Pii was used as a negative control. Expression of all proteins was confirmed in yeast (Figure 2 - figure supplement 1).

Then, we produced the Pikp-HMA\textsuperscript{NK-KE} domain protein via overexpression in \textit{E. coli} and purified it to homogeneity using well-established procedures for these domains (see Materials and Methods, (20,22)). Using SPR, we measured the binding affinity of the Pikp-HMA\textsuperscript{NK-KE} domain to AVR-Pik effectors, alongside wild-type Pikp-HMA, and also Pikm-HMA (Figure 2B, Figure 2 - figure supplement 2,3). Response units (RU) were measured following injection of Pik-HMAs at three different concentrations, after capturing AVR-Pik effectors on a Biacore NTA chip. RUs were then normalised to the theoretical maximum response (R\textsubscript{max}), assuming a 2:1 interaction model for Pikp-HMA and Pikp-HMA\textsuperscript{NK-KE}, and 1:1 for Pikm-HMA, as previously described (22). This data showed an increased binding of Pikp-HMA\textsuperscript{NK-KE}
to AVR-PikE and AVR-PikA compared to wild-type (Figure 2B, Figure 2 - figure supplement 2, Supplementary File 2). The binding of Pikp-HMA\textsuperscript{NK-KE} to the AVR-Pik effectors was also higher compared to Pikm-HMA (Figure 2 - figure supplement 3, Supplementary File 2), correlating with cell death assays (Figure 1 - figure supplement 2). In both cases, statistical analysis has been done for the 40nM analyte (Pik-HMA) data as a representative concentration. Although neither Pikp-HMA nor Pikm-HMA domains show binding to AVR-PikC by SPR, we observe a gain-of-binding of this effector variant with Pikp-HMA\textsuperscript{NK-KE} (Figure 2B, Figure 2 - figure supplement 2,3, Supplementary File 2).

These results show that the Pikp-HMA\textsuperscript{NK-KE} mutant has a higher binding affinity for effectors AVR-PikE and AVR-PikA than wild-type protein. This suggests that the increased binding affinity to the HMA domain correlates with the expanded cell death response in planta (Figure 1B).

The engineered Pikp\textsuperscript{NK-KE} mutant expands association of full-length Pik-1 to effector variants in planta

In addition to interaction with the isolated HMA domain, we tested whether the Asn261Lys/Lys262Glu mutant could expand effector variant binding in the context of the full-length NLR. After generating the mutant in the full-length protein, we co-expressed either Pikp-1 or Pikp-1\textsuperscript{NK-KE} with the AVR-Pik effector variants in N. benthamiana, followed by immunoprecipitation and western blotting to determine effector association.

AVR-PikD shows a robust association with Pikp-1. However, although we observe limited binding for the isolated Pikp-HMA domain by Y2H and SPR, we did not detect association of AVR-PikE and AVR-PikA with the full-length Pikp-1 in planta (Figure 2C). By contrast, we observe clear association of AVR-PikE and AVR-PikA with the Pikp-1\textsuperscript{NK-KE} mutant, albeit with reduced intensity compared to AVR-PikD, correlating with the hierarchical cell death response observed in planta (Figure 1C).
We also detect a very low level of association between full length Pikp-1\textsuperscript{NK-KE} and AVR-PikC (Figure 2C). However, co-expression of Pikp-1\textsuperscript{NK-KE} and AVR-PikC does not result in macroscopic cell death in N. benthamiana (Figure 1C).

These results show that effector variant association to full-length Pikp-1 and Pikp-1\textsuperscript{NK-KE} correlates with the in planta cell death response (Figure 1C).

The effector-binding interface in the Pikp\textsuperscript{NK-KE} mutant adopts a Pikm-like conformation

Having established that the Pikp\textsuperscript{NK-KE} mutant displays an expanded effector recognition profile compared to wild-type, we sort to determine the structural basis of this activity. To this end, we determined crystal structures of Pikp-HMA\textsuperscript{NK-KE} bound to AVR-PikD, and to AVR-PikE. We obtained samples of Pikp-HMA\textsuperscript{NK-KE}/AVR-PikD and Pikp-HMA\textsuperscript{NK-KE}/AVR-PikE complexes by co-expression in E. coli (described in the Materials and Methods and (22)). Each complex was crystallised (see Materials and Methods) and X-ray diffraction data were collected at the Diamond Light Source (Oxford, UK) to 1.6 Å and 1.85 Å resolution respectively. The details of X-ray data collection, structure solution, and completion are given in the Materials and Methods and Table 1.

The overall architecture of these complexes is the same as observed for all Pik-HMA/AVR-Pik effector structures. A key interaction at interface 3, one of the previously defined Pik-HMA/AVR-Pik interfaces (22), involves a Lysine residue (Lys262 in Pikp and Pikm) that forms intimate contacts within a pocket on the effector surface (Figure 3). In order to position this Lysine in the effector pocket, Pikp has to loop-out regions adjacent to this residue, compromising the packing at the interface ((22), Figure 3A (left panel), B (left panel), C and D). By contrast, in Pikm, where the position of the Lysine is shifted one residue to the N-terminus, no looping-out is required to locate the Lysine into the pocket (Figure 3A (right panel), B (right panel), C and D). In the Pikp\textsuperscript{NK-KE} mutant, the position of this key Lysine is shifted one residue to the N-terminus compared to wild-type, and occupies the same
position in the sequence as in Pikm. In the crystal structures of Pikp-HMA\textsuperscript{NK-KE} in complex with either AVR-PikD or AVR-PikE, we see that this region of the HMA adopts a Pikm-like conformation (Figure 3A (middle panel), B (middle panel), C and D), with no looping-out of the preceding structure. This confirms that with the Pikp\textsuperscript{NK-KE} mutant we have resurfaced Pikp to have a more robust, Pikm-like interface in this region.

We found only limited structural perturbations at either of the other previously defined interfaces (interface 1 or 2 (22)) between the AVR-PikD or AVR-PikE effectors bound to Pikp-HMA or Pikp-HMA\textsuperscript{NK-KE} (Figure 3 - figure supplement 1). We therefore conclude that the effects of the Pikp\textsuperscript{NK-KE} mutant on protein function are mediated via altered interactions at interface 3.

**Mutation in AVR-Pik effectors at the engineered binding interface impacts in planta response and in vivo binding**

To further confirm that the engineered binding interface is responsible for the expanded recognition of AVR-PikE and AVR-PikA by Pikp\textsuperscript{NK-KE}, we used mutants in the effectors at interface 2 (AVR-PikD\textsuperscript{H46E}) and interface 3 (AVR-PikD,E,A\textsuperscript{E53R}), which have previously been shown to impact interactions and in planta responses in wild-type NLR alleles (22). We tested whether these mutants affected the cell death response in *N. benthamiana*, and interactions between effectors and Pikp-HMA\textsuperscript{NK-KE} (Y2H) and between effectors and full length Pikp\textsuperscript{NK-KE} (in planta co-immunoprecipitation (co-IP)).

Firstly, we investigated the impact of mutation at interface 2 using the AVR-PikD\textsuperscript{H46E} mutant. Similar to Pikp, we found that cell death in *N. benthamiana* is essentially blocked when co-expressing Pikp\textsuperscript{NK-KE} with this mutant (AVR-PikD\textsuperscript{H46E}), suggesting that the engineered NLR is still reliant on this interface for response (Figure 4A, Figure 4 - figure supplement 1). Intriguingly, Y2H shows that the AVR-PikD\textsuperscript{H46E} mutant displays some interaction with Pikp-HMA\textsuperscript{NK-KE} (Figure 4B), similar to this
mutant’s interaction with Pikm-HMA (22), although this interaction is barely observed by co-IP with the full length NLR (Figure 4C).

Secondly, we investigated the impact of mutations at interface 3 using the Glu53Arg (E53R) mutant in AVR-PikD, AVR-PikE and AVR-PikA. We found that the AVR-PikD\textsuperscript{E53R} mutant has essentially no effect on recognition of the effector by Pikp\textsuperscript{NK-KE} in *N. benthamiana*, and little/no effect on interaction with Pikp-HMA\textsuperscript{NK-KE}, or full-length Pikp\textsuperscript{NK-KE} (Figure 4A,B,C, Figure 4 – figure supplement 1). By contrast, the equivalent mutation in AVR-PikE and AVR-PikA restricted the cell death response in *N. benthamiana*, reduced the binding to Pikp-HMA\textsuperscript{NK-KE} in Y2H (as shown by the reduced blue colouration) and a less intense band is observed for the effector following Pikp\textsuperscript{NK-KE} co-IP (Figure 4A,B,C). Expression of all proteins in yeast was confirmed by western blot (Figure 4 – figure supplement 2).

These results support that whilst interactions across interface 2 remain important for the Pikp\textsuperscript{NK-KE} interaction with AVR-Pik effectors, it is the altered interaction at interface 3, as observed in the structures, that is responsible for the expanded recognition profile of this engineered mutant.
Discussion

Plants, including food crops, are under continuous threat from pathogens and pests, and new solutions to control disease are required. While largely elusive to date, engineering plant NLR-type intracellular immune receptors has potential as a method for improving disease resistance breeding (4,6). NLR integrated domains are a particularly attractive target for protein engineering as they directly interact with pathogen effectors (or host effector targets). Further, where tested, binding affinities in vitro correlate with in planta immunity phenotypes (20,22,27), allowing biochemical and structural techniques to directly inform NLR design.

Here we show that the recognition profile of the rice NLR Pikp can be expanded to different AVR-Pik variants by engineering the binding interface between these proteins. This strengthens the hypothesis that tighter binding affinity between effectors and integrated HMA domains correlates with increased immune signalling in plants. This was previously shown for both natural alleles of Pik (20,22), and also for Pia (27), but is now also shown for an engineered NLR. We propose this may be a general model for integrated domains that directly bind effectors.

Natural variation in Pik NLRs has given rise to different effector recognition profiles, and contribution from different binding interfaces was suggested to underpin this phenotype (22). In particular, a more favourable interaction at one interface (interface 3) in Pikm, compared to Pikp, was concluded to have evolved to compensate for changes in binding at a different site (interface 2). Here, through mutation of residues in Pikp (forming Pikp\textsuperscript{NK,KE}), we have combined favourable interfaces from Pikp and Pikm into a single protein. This has resulted in an expanded recognition phenotype for effector binding and response in planta. In vitro, the binding of Pikp\textsuperscript{NK,KE-HMA} to effector variants is consistently higher than that of Pikm-HMA (Figure 2 - figure supplement 3). This is likely underpinned by differences in a cluster of key residues within interface 2 (spatially equivalent residues Val222, Lys228, and Glu230 in Pikp, and Ala223, Gln229, and Val231 in Pikm) forming more energetically favourable contacts, including water-mediated hydrogen bonds. However, despite a positive trend (significant in some cases), the
cell death mediated by Pikm and Pikp\textsuperscript{NK-KE} in planta are comparable (Figure 1 - figure supplement 2,4).

While the Pikp\textsuperscript{NK-KE} mutant did not deliver a cell death response in *N. benthamiana* to the stealthy AVR-PikC effector variant, it did show a gain-of-binding in vitro, and weak binding in Y2H and in in planta co-IP. It is surprising that the gain-of-binding for AVR-PikC in vitro does not correlate directly with gain of in vivo interactions or cell death response in *N. benthamiana*, as it does with AVR-PikA. However, we hypothesise that this gain-of-binding for AVR-PikC observed in vitro is not sufficiently robust, especially in the context of the full-length NLR, for binding or triggering immune signalling. However, this work sets the scene for future interface engineering experiments that may further improve the response profiles of Pik NLRs to currently unrecognised effector variants. It also requires future work to test the disease resistance profile of *M. oryzae* strains carrying the different effector variants in rice expressing the engineered receptor.

The integrated HMA domain in the NLR RGA5 (the sensor of the Pia NLR pair in rice), binds to *M. oryzae* effectors AVR1-CO39 and AVR-Pia via a different interface, and it has been suggested that these binding sites are mutually exclusive (27). This raises the possibility that an HMA domain could be engineered to bind and respond to multiple effectors (27). Recently, the Pikp-HMA domain was shown to interact with AVR-Pia at the same interface as used by the RGA5-HMA domain, and this likely underpins partial resistance to *M. oryzae* expressing AVR-Pia in planta (31). This presents a starting point for using Pikp as a chassis for such studies. While it remains to be seen whether any such resurfaced HMA domain can bind to multiple effectors, these studies suggest this has potential as a novel approach.

Plant breeding is required to provide new genetic solutions to disease resistance in crops. This is necessary to limit the environmental and social damage caused by pesticides, and to deal with changes in climate and globalisation of agriculture that result in the spread of pathogens and pests into new environments (32-34). Classical breeding for disease resistance has been limited by issues such as linkage drag and
hybrid incompatibility, as also seen in model plant species (35). Novel molecular
approaches such as engineering “decoys” (12) and protein resurfacing, as described
here, combined with modern transformation (36) and breeding pipelines (37), offers
the opportunity for more targeted approaches to disease resistance breeding. These
will complement other emerging technologies in NLR identification (38) and NLR
stacking (4) as methods to develop improved crops for the future.
Accession codes

Protein structures, and the data used to derive these, have been deposited at the Protein DataBank (PDB) with accession codes 6R8K (Pikp-HMA\textsuperscript{NK-KE}/AVR-PikD) and 6R8M (Pikp-HMA\textsuperscript{NK-KE}/AVR-PikE).

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**Materials and Methods**

**Gene cloning**

For in vitro studies, Pikp-HMA\(^{NK-KE}\) (encompassing residues 186 to 263) was amplified from WT Pikp-HMA by introducing the mutations in the reverse primer, followed by cloning into pOPINM (39). Wild-type Pikp-HMA, Pikm-HMA, and AVR-Pik expression constructs used in this study are as described in (22).

For Y2H, we cloned Pikp-HMA\(^{NK-KE}\) (as above) into pGBKT7 using an In-Fusion cloning kit (Takara Bio USA), following the manufacture’s protocol. Wild-type Pikp-HMA domain in pGBKT7 and AVR-Pik effector variants in pGADT7 used were generated as described in (22).

For protein expression in planta, Pikp-HMA\(^{NK-KE}\) domain was generated using site directed mutagenesis by introducing the mutations in the reverse primer. This domain was then assembled into a full-length construct using Golden Gate cloning (40) and into the plasmid pICH47742 with a C-terminal 6xHis/3xFLAG tag. Expression was driven by the *A. tumefaciens* Mas promoter and terminator. Full-length Pikp-1, Pikp-2, and AVR-Pik variants used were generated as described in (22).

All DNA constructs were verified by sequencing.

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

pOPINM constructs encoding Pikp-HMA, Pikm-HMA and Pikp-HMA\(^{NK-KE}\) were produced in *E. coli* SHuffle cells (41) using the same protocol described in (22). Cell cultures were grown in auto induction media (42) at 30°C for 5 – 7hrs and then at 16°C overnight. 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, 20 mM imidazole supplemented with EDTA-free protease inhibitor tablets (Roche). Cells were sonicated and, following centrifugation at 40000xg for 30 min, 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 (50 mM Tris-
HCl pH7.5, 500 mM NaCl, 50 mM Glycine, 5% (vol/vol) glycerol, 500 mM imidazole) and directly injected onto a Superdex 75 26/60 gel filtration column pre-equilibrated 20mM HEPES pH 7.5, 150 mM NaCl. Purification tags were removed by incubation with 3C protease (10 μg/mg fusion protein) followed by passing through tandem Ni²⁺-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 20 mM HEPES pH 7.5, 150 mM NaCl.

AVR-Pik effectors, with either a 3C protease-cleavable N-terminal SUMO or MBP tag, and a non-cleavable C-terminal 6xHis tag, were produced in and purified from *E. coli* SHuffle cells as previously described (20,22). All protein concentrations were determined using a Direct Detect® Infrared Spectrometer (Merck).

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

Pikp-HMA<sub>NK-KE</sub> was co-expressed with AVR-PikD or AVR-PikE effectors in *E. coli* SHuffle cells following co-transformation of pOPINM:Pikp-HMA<sub>NK-KE</sub> and pOPINA:AVR-PikD/E (which were prepared as described in (22)). Cells were grown in autoinduction media (supplemented with both carbenicillin and kanamycin), harvested, and processed as described in (22). Protein concentrations were measured using a Direct Detect® Infrared Spectrometer (Merck).

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

To detect protein–protein interactions between Pik-HMAs and AVR-Pik effectors by Yeast Two-Hybrid, we used the Matchmaker® Gold System (Takara Bio USA). Plasmid DNA encoding Pikp-HMA<sub>NK-KE</sub> in pGBK7, generated in this study, was co-transformed into chemically competent Y2HGol cells (Takara Bio, USA) with the individual AVR-Pik variants or mutants in pGADT7 described previously (22). Single colonies grown on selection plates were inoculated in 5 ml of SD<sup>-Leu-Trp</sup> overnight at 30°C. Saturated culture was then used to make serial dilutions of OD<sub>600</sub> 1, 1^-1, 1^-2, 1^-3, respectively. 5 μl of each dilution was then spotted on a SD<sup>-Leu-Trp</sup> plate as a growth control, and on a SD<sup>-Leu-Trp-Ade-His</sup> plate containing X-α-gal and
supplemented with Aureobasidin A (Takara Bio, USA). Plates were imaged after incubation for 60 - 72 hr at 30°C. Each experiment was repeated a minimum of 3 times, with similar results.

To confirm protein expression in yeast, total protein extracts from transformed colonies were produced by boiling the cells 10 minutes in LDS Runblue® sample buffer. Samples were centrifugated and the supernatant was subjected to SDS-PAGE gels prior to western blotting. The membranes were probed with anti-GAL4 DNA-BD (Sigma) for the HMA domains in pGBKT7 and anti-GAL4 activation domain (Sigma) antibodies for the AVR-Pik effectors in pGADT7.

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

Surface plasmon resonance (SPR) experiments were performed on a Biacore T200 system (GE Healthcare) using an NTA sensor chip (GE Healthcare). The system was maintained at 25°C, and a flow rate of 30 µl/min was used. All proteins were prepared in SPR running buffer (20 mM HEPES pH 7.5, 860 mM NaCl, 0.1% Tween 20). C-terminally 6xHis-tag AVR-Pik variants were immobilised on the chip, giving a response of 200 ± 100. The sensor chip was regenerated between each cycle with an injection of 30 µl of 350 mM EDTA.

For all the assays, the level of binding was expressed as a percentage of the theoretical maximum response ($R_{max}$) normalized for the amount of ligand immobilized on the chip. The cycling conditions were the same as used in (22). 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 (43). SPR data was exported and plotted using R v3.4.3 (https://www.r-project.org/) and the function ggplot2 (Wickham, H., 2009). Each experiment was repeated a minimum of 3 times, including internal repeats, with similar results. The proteins used came from three independent preparations for the HMA domains and two independent preparations of the AVR-Pik effectors.

**Protein-protein interaction: In planta co-immunoprecipitation (co-IP)**
Transient gene-expression in planta for Co-IP was performed by delivering T-DNA constructs with *Agrobacterium tumefaciens* GV3101 strain into 4-week old *N. benthamiana* plants grown at 22–25°C with high light intensity. *A. tumefaciens* strains carrying Pikp-1 or Pikp-1\(^{\text{NK-KE}}\) were mixed with strains carrying the AVR-Pik effector, at OD\(_{600}\) 0.2 each, in agroinfiltration medium (10 mM MgCl\(_2\), 10 mM 2-(N-morpholine)-ethanesulfonic acid (MES), pH 5.6), supplemented with 150 µM acetylsyringone. For detection of complexes in planta, leaf tissue was collected 3 days post infiltration (dpi), frozen, and ground to fine powder in liquid nitrogen using a pestle and mortar. Leaf powder was mixed with 2 times weight/volume ice-cold extraction buffer (10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 2% w/v PVPP, 10 mM DTT, 1x protease inhibitor cocktail (Sigma), 0.1% Tween 20 (Sigma)), centrifuged at 4,200g/4°C for 20-30 min, and the supernatant was passed through a 0.45µm Minisart® syringe filter. The presence of each protein in the input was determined by SDS-PAGE/western blot. Pik-1 and AVR-Pik effectors were detected probing the membrane with anti-FLAG M2 antibody (SIGMA) and anti c-Myc monoclonal antibody (Santa Cruz), respectively. For immunoprecipitation, 1.5ml of filtered plant extract was incubated with 30 µl of M2 anti-FLAG resin (Sigma) in a rotatory mixer at 4°C. After three hours, the resin was pelleted (800g, 1 min) and the supernatant removed. The pellet was washed and resuspended in 1ml of IP buffer (10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% Tween 20 (Sigma)) and pelleted again by centrifugation as before. Washing steps were repeated 5 times. Finally, 30 µl of LDS Runblue® sample buffer was added to the agarose and incubated for 10 min at 70°C. The resin was pelleted again, and the supernatant loaded on SDS-PAGE gels prior to western blotting. Membranes were probed with anti-FLAG M2 (Sigma) and anti c-Myc (Santa Cruz) monoclonal antibodies.

*N. benthamiana* cell death assays

*A. tumefaciens* GV3101 carrying Pikp-1, Pikm-1, or Pikp-1\(^{\text{NK-KE}}\) were resuspended in induction media (10 mM MES pH 5.6, 10 mM MgCl\(_2\) and 150 µM acetylsyringone) and mixed with Pikp-2 (or Pikm-2 in Pikm cell death assay), AVR-Pik effectors, and
P19 at OD<sub>600</sub> 0.4, 0.4, 0.6 and 0.1, respectively. 4-weeks old <i>N. benthamiana</i> leaves were infiltrated using a needleless syringe. Leaves were collected at 5 dpi to measure UV autofluorescence (a proxy for cell death) or ion leakage.

**Cell death scoring: UV autofluorescence**

Detached leaves were imaged at 5 dpi from the abaxial side of the leaves for UV fluorescence images. Photos were taken using a Nikon D4 camera with a 60mm macro lens, ISO set 1600 and exposure ~10secs at F14. The filter is a Kodak Wratten No.8 and white balance is set to 6250 degrees Kelvin. Blak-Ray® longwave (365nm) B-100AP spot light lamps are moved around the subject during the exposure to give an even illumination. Images shown are representative of three independent experiments, with internal repeats. The cell death index used for scoring is as presented previously (20). Dotplots were generated using R v3.4.3 (https://www.r-project.org/) and the graphic package ggplot2 (Wickham, H., 2009). The size of the centre dot at each cell death value is directly proportional to the number of replicates in the sample with that score. All individual data points are represented as dots.

**Cell death scoring: Ion leakage**

For ion leakage quantification, plants were infiltrated with the relevant constructs on two different leaves. After 5 dpi, leaves were detached and two leaf discs with a diameter of 8mm (one disc per leaf spot) were collected and floated in 1.5mL of Milli-Q water. Conductivity (µS/cm) was measured immediately after transferring the leaf disc to water (time zero) using a LAQUAtwin EC-33 conductivity meter (Horiba UK Ltd). Leaf discs were then incubated for six hours at room temperature with gentle shaking before measuring the final conductivity. The assay was carried out in six biological replicates with a total of forty technical replicates (2 discs x 40
Conductivity data for each sample was exported and plotted using R v3.4.3 (https://www.r-project.org/) and the function ggplot2 (Wickham, H., 2009).

**Crystallization, data collection and structure solution**

For crystallization, Pikp-HMA$^{NK-KE}$ in complex with AVR-PikD or AVR-PikE were concentrated following gel filtration. Sitting drop vapor diffusion crystallization trials were set up in 96 well plates, using an Oryx nano robot (Douglas Instruments, United Kingdom). Plates were incubated at 20°C, and crystals typically appeared after 24 - 48 hours. For data collection, all crystals were harvested from the Morpheus® HT-96 screen (Molecular Dimensions), and snap-frozen in liquid nitrogen. Crystals used for data collection appeared from the following conditions: (i) Pikp-HMA$^{NK-KE}$/AVR-PikD (10 mg/ml), Morpheus® HT-96 condition D4 [0.12 M Alcohols (0.2 M 1,6-Hexanediol; 0.2 M 1-Butanol; 0.2 M 1,2-Propanediol; 0.2 M 2-Propanol; 0.2 M 1,4-Butanediol; 0.2 M 1,3-Propanediol); 0.1 M Buffer system 1 (1 M 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)]; (ii) Pikp-HMA$^{NK-KE}$/AVR-PikE (15 mg/ml), Morpheus® HT-96 condition A8 [0.06M Divalent (0.3 M Magnesium chloride hexahydrate; 0.3 M Calcium chloride dihydrate); 0.1M Buffer system 2 (Sodium HEPES; MOPS (acid)) pH 7.5; 37.5%v/v Precipitant mix 4 (25%v/v MPD; 25%v/v PEG 1000; 25%v/v PEG3350)].

X-ray data sets were collected at the Diamond Light Source using beamline i03 (Oxford, UK). The data were processed using the xia2 pipeline (44) and CCP4 (45). The structures were solved by molecular replacement using PHASER (46) and the coordinates of AVR-PikD and a monomer of Pikp-HMA from PDB entry 6G10. The final structures were obtained through iterative cycles of manual rebuilding and refinement using COOT (47) and REFMAC5 (48), as implemented in CCP4 (45). Structures were validated using the tools provided in COOT and MOLPROBITY (49).
Statistical analyses

Qualitative cell death scoring from autofluorescence was analysed using estimation methods (50) and visualised with estimation graphics using the besthr R library (51). Briefly, in this process all autofluorescence (cell death) scores in samples under comparison are ranked, irrespective of sample. The mean ranks of the control and test sample are taken and a bootstrap process is begun on ranked test data, in which samples of equal size to the experiment are taken with replacement and the mean rank calculated. After 1000 bootstrap sample rank means have been calculated, a distribution of the mean ranks is drawn and its 2.5 and 97.5 quantile calculated. If the mean of the control data is outside of these boundaries the control and test means are considered different. Quantitative data from ion leakage (cell death) and SPR assays were analysed by preparing a linear mixed effects model of sample on ion leakage/SPR and post-hoc comparisons performed for sample contrasts using Tukey’s HSD method in the R package nlme (52) and lsmeans (53).
Table 1: Data Collection and Refinement statistics

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*The highest resolution shell is shown in parenthesis.

*As calculated by Aimless, †As calculated by Refmac5, **As calculated by MolProbity
**Figures**

**Figure 1 - Structure-informed engineering expands Pikp-mediated effector recognition in N. benthamiana.** (A) Sequence alignment of Pikp-1 and Pikm-1 HMA domains. Secondary structure features of the HMA fold are shown above, and the residues located to binding interfaces are as coloured. Key residues from interface 2 and interface 3 involved in this study are highlighted in red. (B) Representative leaf images showing Pikp (left) or Pikp-1NK-KE (right)-mediated cell death to AVR-Pik variants as autofluorescence under UV light. (C) Autofluorescence intensity is scored as previously described (20,22). Cell death assay scoring is represented as dot plots for Pikp and PikpNK-KE (blue and purple respectively). For each sample, all the data points are represented as dots with a distinct colour for each of the three biological replicates; these dots are jittered about the cell death score for visualisation purposes. The size of the centre dot at each cell death value is directly proportional to the number of replicates in the sample with that score. The total number of repeats was 80. Data for Pikp has been previously shown (22), but was acquired at the same time as PikpNK-KE. Estimation methods, used to visualise differences in the data sets, are shown in **Figure 1 - figure supplement 3.** (D) Conductivity measurements showing ion leakage as a quantitative measure of cell death. The centre line represents the median, the box limits are the upper and lower quartiles, the whiskers extend to the largest value within Q1 - 1.5x the interquartile range (IQR) and the smallest value within Q3 + 1.5x IQR. All the data points are shown as dots with distinct colours for each biological replicate. For each experiment, six biological replicates with 5 or 10 internal repeats were performed (total data points = 40). “p” is the p-value obtained from statistical analysis and Tukey’s HSD.

**Figure 1 - figure supplement 1 - Mutations at interface 2 of Pikp-1 HMA domain compromise response to AVR-Pik effectors.** (A) Cell death assay scoring represented as dot plots for Pikp-1 mutants on HMA interface 2 and 3. For each sample, all the data points are represented as dots with a distinct colour for each of the three biological replicates; these dots are jittered about the cell death score for
visualisation purposes. The size of the central dot at each cell death value is proportional to the number of replicates of the sample with that score. The number of repeats was 18 for each mutant. (B) Statistical analysis by estimation methods of the cell death assay for Pikp-1 mutants. The left panel represents the ranked data (dots) for each effector, and their corresponding mean (dotted line). The size of the dots is proportional to the number of observations with that value. The right panel shows the distribution of 1000 bootstrap sample rank means for each effector. The blue areas represent the 0.025 and 0.975 percentiles of the distribution. A sample (effector) score is considered significantly different from the control (EV) when the control rank mean (dotted line on the left) falls beyond the blue regions of the effector mean distribution. When the rank mean for different effectors have the same value, only one dotted line is visible (EV, AVR-E and AVR-C for Pikp and K228Q, and EV, AVR-A and AVR-C for V222A). (C) Western blot analysis confirming similar levels of Pik-1 protein accumulation in N. benthamiana. The asterisks mark the Pik-1 band, PS = Ponceau Stain.

Figure 1 - figure supplement 2 - Response of Pikp\textsubscript{NK-KE} to AVR-Pik effectors compared to Pikm. (A) Cell death autofluorescence scoring represented as dot plots for Pikm and Pikp\textsubscript{NK-KE} (yellow and purple respectively). The number of repeats was 80 and 90 for Pikp\textsubscript{NK-KE} and Pikm, respectively. For each sample, all the data points are represented as dots with a distinct colour for each of the three biological replicates; these dots are jittered about the cell death score for visualisation purposes. The size of the central dot at each cell death value is proportional to the number of replicates of the sample with that score. Data for Pikm has been previously shown (22), but was acquired at the same time as Pikp\textsubscript{NK-KE}. Estimation methods, used to visualise differences in the data sets, are shown in Figure 1, figure supplement 4. (B) Conductivity measurements showing ion leakage as a quantitative measure of cell death. The centre line represents the median, the box limits are the upper and lower quartiles, the whiskers extend to the largest value within Q1 - 1.5x the interquartile range (IQR) and the smallest value within Q3 +
1.5x IQR. All the data points are shown as dots with distinct colours for each biological replicate. For each experiment, six biological replicates with 5 or 10 internal repeats were performed (total data points = 40). “p” is the p-value obtained from statistical analysis and Tukey’s HSD.

Figure 1 - figure supplement 3 – Estimation graphics for cell death, Pikp vs Pikp\(^{NK-KE}\). Statistical analysis by estimation methods of the cell death assay for Pikp and Pikp\(^{NK-KE}\). For each effector, the panel on the left represents the ranked data (dots) for each NLR, and their corresponding mean (dotted line). The size of the dots is proportional to the number of observations with that specific value. The panel on the right shows the distribution of 1000 bootstrap sample rank means for Pikp\(^{NK-KE}\). The blue areas represent the 0.025 and 0.975 percentiles of the distribution. The response of Pikp and Pikp\(^{NK-KE}\) are considered significantly different if the Pikp rank mean (dotted line, left panel) falls beyond the blue regions of Pikp\(^{NK-KE}\) mean distribution.

Figure 1 - figure supplement 4 – Estimation graphics for cell death, Pikm vs Pikp\(^{NK-KE}\). Statistical analysis by estimation methods of the cell death assay for Pikm and Pikp\(^{NK-KE}\). For each effector, the panel on the left represents the ranked data (dots) for each NLR, and their corresponding mean (dotted line). The size of the dots is proportional to the number of observations with that specific value. The panel on the right shows the distribution of 1000 bootstrap sample rank means for Pikp\(^{NK-KE}\). The blue areas represent the 0.025 and 0.975 percentiles of the distribution. The response of Pikm and Pikp\(^{NK-KE}\) are considered significantly different if the Pikp rank mean (dotted line, left panel) falls beyond the blue regions of Pikp\(^{NK-KE}\) mean distribution.

Figure 2 - Pikp\(^{NK-KE}\) shows increased binding to effector variants in vivo and in vitro compared to wild type. (A) Yeast-Two-Hybrid assay of Pikp-HMA and Pikp-
HMA\textsuperscript{NK-KE} with AVR-Pik alleles. Control plate for yeast growth is on the left, with quadruple dropout media supplemented with X-α-gal and increasing concentrations of Aureobasidin A on the right for each combination of HMA/AVR-Pik. The unrelated \textit{M. oryzae} effector AVR-Pii was used as a negative control. Growth and development of blue colouration in the selection plate are both indicative of protein:protein interaction. HMA domains were fused to the GAL4 DNA binding domain, and AVR-Pik alleles to the GAL4 activator domain. Each experiment was repeated a minimum of three times, with similar results.

(B) Box plots showing \%R\textsubscript{max} for Pikp-HMA and Pikp-HMA\textsuperscript{NK-KE} with the AVR-Pik effectors alleles at HMA concentration of 40 nM as measured by surface plasmon resonance. Pikp-HMA and Pikp-HMA\textsuperscript{NK-KE} are represented by blue and purple boxes, respectively. The centre line represents the median, the box limits are the upper and lower quartiles, the whiskers extend to the largest value within Q1 \(-1.5\times\) the interquartile range (IQR) and the smallest value within Q3 \(+1.5\times\) IQR. All the data points are represented as dots with distinct colours for each biological replicate. For each experiment, three biological replicates with three internal repeats were performed. “p” is the p-value obtained from statistical analysis and Tukey’s HSD. For results of experiments with 4 and 100 nM HMA protein concentration see Figure 2 - figure supplement 2.

(C) Co-immunoprecipitation of full length Pikp-1 and Pikp-1\textsuperscript{NK-KE} with AVR-Pik variants. N-terminally 4xMyc tagged AVR-Pik effectors were transiently co-expressed with Pikp-1:6xHis3xFLAG (left) or Pikp-1\textsuperscript{NK-KE}:6xHis3xFLAG (right) in \textit{N. benthamiana}. Immunoprecipitates (IPs) obtained with anti-FLAG antiserum, and total protein extracts, were probed with appropriate antisera. Dashed line indicates a crop site on the same blot used to compose the figure. Each experiment was repeated at least three times, with similar results. The asterisks mark the Pik-1 band, PS = Ponceau Stain.

Figure 2 - figure supplement 1 - Western blot confirming accumulation of proteins in yeast. Yeast lysate was probed for the expression of HMA domains with anti-GAL4 DNA binding domain (BD) and AVR-Pik/AVR-Pii effectors anti-GAL4 activation domain (AD). Total extract was coloured with Ponceau Stain (PS).
experiment was repeated a minimum of 3 times, with similar results. PS = Ponceau

Figure 2 - figure supplement 2 - Binding of the Pikp-HMA\textsuperscript{NK-KE} domain to the
AVR-Pik effectors is consistently higher compared to Pikp-HMA. \%R\textsubscript{max} of Pikp-
HMA and Pikp-HMA\textsuperscript{NK-KE} with the AVR-Pik effectors alleles with HMA
concentrations of 4nM (left) and 100nM (right). The centre line represents the
median, the box limits are the upper and lower quartiles, the whiskers extend to the
largest value within Q1 - 1.5× the interquartile range (IQR) and the smallest value
within Q3 + 1.5× IQR. All the data points are represented as dots with distinct
colours for each biological replicate. For each experiment, three biological replicates
with three internal replicates were performed.

Figure 2 - figure supplement 3 - Binding of the Pikp-HMA\textsuperscript{NK-KE} domain to the
AVR-Pik effectors is consistently higher compared to Pikm-HMA. Surface
plasmon resonance \%R\textsubscript{max} values for Pikm-HMA and Pikp-HMA\textsuperscript{NK-KE} with the AVR-
Pik effectors alleles. Pikm-HMA and Pikp-HMA\textsuperscript{NK-KE} results are represented by
yellow and purple boxes, respectively. The centre line represents the median, the box
limits are the upper and lower quartiles, the whiskers extend to the largest value
within Q1 - 1.5× the interquartile range (IQR) and the smallest value within Q3 +
1.5× IQR. All the data points are represented as dots with distinct colours for each
biological replicate. For each experiment, we performed at least two biological
replicates with three internal repeats. Results using HMA protein concentration of 4,
40 and 100 nM are plotted in the left, middle (“p” is the p-value obtained from
statistical analysis and Tukey’s HSD) and right panels, respectively.

Figure 3 - The Pikp\textsuperscript{NK-KE}-HMA mutant adopts a Pikm-like conformation at the
effector binding interface. Schematic view of the different conformations adopted
by Pikp-HMA, Pikp-HMA\textsuperscript{NK-KE} and Pikm-HMA at interface 3 in complex with AVR-
PikD or AVR-PikE. In each panel, the effector is shown as sticks with the molecular surface also shown and coloured as labelled. Pik-HMA residues are coloured as labelled and shown in the Cα-worm with side-chain representation. (A) Schematic of Pikp-HMA (left), Pikp-HMA\textsuperscript{NK-KE} (middle) and Pikm-HMA (right) bound to AVR-PikD. Important residues involved in HMA/effector interaction are labelled as shown. (B) Schematic of HMA residues as for panel (A), but bound to AVR-PikE. (C) Superposition showing Pikp-HMA, Pikp-HMA\textsuperscript{NK-KE} and Pikm-HMA chains (coloured in blue, purple and yellow, respectively) bound to AVR-PikD. For clarity, only the Lys-261/262 side chain is shown. (D) Superposition as described before, but bound to AVR-PikE.

**Figure 3 - figure supplement 1 - Interface 2 is essentially identical in the complexes of Pikp-HMA and Pikp-HMA\textsuperscript{NK-KE} bound to AVR-PikD or AVR-PikE.**

Schematic view of the conformations adopted by Pikp-HMA and Pikp-HMA\textsuperscript{NK-KE} at interface 2 in complex with AVR-PikD or AVR-PikE. In each panel, the effector is shown as sticks with the molecular surface also shown and coloured as labelled. Pik-HMA residues are coloured as labelled and shown in the Cα-worm with side-chain representation. The structures were overlaid on the effectors. (A) Pikp-HMA and Pikp-HMA\textsuperscript{NK-KE} (coloured in blue and purple, respectively) bound to AVR-PikD (light and dark green). (B) Pikp-HMA and Pikp-HMA\textsuperscript{NK-KE} bound to AVR-PikE (light and dark blue).

**Figure 4 - Mutation of AVR-Pik effectors at the engineered binding interface compromises binding and response.** (A) Left - a representative leaf image showing Pikp-\textsuperscript{1NK-KE}-mediated cell death to AVR-Pik variants and mutants as autofluorescence under UV light (the AVR-PikD\textsuperscript{H46E} mutant is located to interface 2, while the AVR-PikD\textsuperscript{E53R}, AVR-PikE\textsuperscript{E53R}, and AVR-PikA\textsuperscript{E53R} mutants are located to interface 3). Autofluorescence intensity is scored as in Figure 1. Right - Pikp\textsuperscript{NK-KE} cell death assay quantification is represented as dot plots. For each sample the data points are represented as dots with a distinct colour for each of the three biological
replicates; these dots are jittered about the cell death score for visualisation purposes. The size of the central dot at each cell death value is proportional to the number of replicates of the sample with that score. The number of repeats was 90. 

(B) Yeast-Two-Hybrid assay of Pikp-HMA$^{NK-KE}$ with AVR-Pik variants and mutants. Control plate for yeast growth is on the left with quadruple dropout media supplemented with X-α-gal and increasing concentrations of Aureobasidin A on the right for each combination of HMA/AVR-Pik. The unrelated $M.~oryzae$ effector AVR-Pii was used as a negative control. Growth and development of blue colouration in the selection plate are both indicative of protein:protein interaction. HMA domains were fused to the GAL4 DNA binding domain, and AVR-Pik alleles to the GAL4 activator domain. Each experiment was repeated a minimum of three times, with similar results. (C) Co-immunoprecipitation of full length Pikp-1$^{NK-KE}$ with AVR-Pik variants and mutants. N-terminally 4xMyc tagged AVR-Pik effectors were transiently co-expressed with Pikp-1$^{NK-KE}$:6xHis3xFLAG in $N.~benthamiana$ leaves. Immunoprecipitates (IPs) obtained with anti-FLAG antiserum, and total protein extracts, were probed with appropriate antisera. Each experiment was repeated at least three times, with similar results. The asterisks mark the Pik-1 band, PS = Ponceau Stain.

Figure 4 - figure supplement 1 - Estimation graphics for cell death, Pikp$^{NK-KE}$ vs effector mutants. Statistical analysis by estimation methods of the cell death assay for Pikp$^{NK-KE}$ with AVR-Pik variants and mutants. The response to each mutant is compared with the response to the corresponding WT variant. For each couple, the panel on the left represents the ranked data (dots) for each effector, and their corresponding mean (dotted line). The size of the dots is proportional to the number of observations with that specific value. The panel on the right shows the distribution of 1000 bootstrap sample rank means for the mutant. The blue areas represent the 0.025 and 0.975 percentiles of the distribution. The response to Pikp$^{NK-KE}$ for WT and the effector mutants are considered significantly different if the WT
Figure 4 - figure supplement 2 - Western blot analysis confirming accumulation of proteins in yeast. Yeast lysate was probed for the expression of HMA domain with anti-GAL4 DNA binding domain (BD) and AVR-Pik/AVR-Pii effectors anti-GAL4 activation domain (AD). The experiment was repeated a minimum of 3 times, with similar results. CBS = Coomassie Blue Stain. In each case the effector mutants consistently accumulate to higher levels than the wild-type proteins, but result in weaker readouts of protein:protein interaction in the Y2H assay.
**Supplementary Files**

**Supplementary File 1:** Table of p-values for all pairwise comparisons of the ion leakage data in *N. benthamiana*. Underlined values are those presented in the respective Figures.

**Supplementary File 2:** Table of p-values for all pairwise comparisons of the SPR data including Pikp and Pikp^{NK-KF}. Underlined values are those presented in the respective Figures.

**Source Data Files**

**Figure 1 – source data 1:** Cell death scoring data used in the preparation of Figure 1C.

**Figure 1 – source data 2:** Conductivity measurements used in the preparation of Figure 1D and Figure 1 – figure supplement 2B.

**Figure 1 – source data 3:** Cell death scoring data used in the preparation of Figure 1 – figure supplement 1A.

**Figure 1 – source data 4:** Cell death scoring data used in the preparation of Figure 1 – figure supplement 2A.

**Figure 2 – source data 1:** Surface Plasmon Resonance measurements used in the preparation of Figure 2B.
Figure 2 – source data 2: Surface Plasmon Resonance measurements used in the preparation of Figure 2 – figure supplement 2, left panel.

Figure 2 – source data 3: Surface Plasmon Resonance measurements used in the preparation of Figure 2 – figure supplement 2, right panel.

Figure 2 – source data 4: Surface Plasmon Resonance measurements used in the preparation of Figure 2 – figure supplement 3.

Figure 4 – source data 1: Cell death scoring data used in the preparation of Figure 4A.


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