Directed evolution of a plant immune receptor for broad spectrum recognition of pathogen effectors

Authors: Ellen Y. Rim[1*], Oscar D. Garrett[1†], Alexander J. Howard[1†], Yejin Shim[1], Yuanyuan Li[2], Jonathan E. Van Dyke[3], Ryan C. Packer[1], Nguyen Ho[1], Rashmi S. Jain[1], Valley J. Stewart[1], Savithramma P. Dinesh-Kumar[2], James H. Notwell[1], Pamela C. Ronald[1,4,5*]

Affiliations:

[1]Department of Plant Pathology and the Genome Center, University of California, Davis, CA, 95616, USA.

[2]Department of Plant Biology and the Genome Center, University of California, Davis, CA, 95616, USA.

[3]Flow Cytometry Shared Resource Laboratory, University of California, Davis, CA, 95616, USA.

[4]Joint BioEnergy Institute, Emeryville, CA 94608, USA.

[5]Innovative Genomics Institute (IGI), University of California, Berkeley, CA 94720, USA.

*Corresponding authors. Email: yrim@ucdavis.edu (E.Y.R.); pcronald@ucdavis.edu (P.C.R.)

†These authors contributed equally to this work.

Abstract:

Rapid development of immune receptors that protect crops from emerging pathogens is a paramount goal. We applied high throughput directed evolution to the ligand binding domain of the rice immune receptor Pik1 to engineer affinity for a wide range of effectors from the fast-evolving fungal pathogen *Magnaporthe oryzae.* Engineered domains showed affinity for variants of the *M. oryzae* effector Avr-Pik that previously evaded detection by known rice alleles of Pik1. Additional rounds of mutagenesis and selection yielded a Pik1 domain that binds all tested Avr-Pik variants as well as the evolutionarily divergent effector AvrPiz-t. Our results demonstrate the potential of directed evolution to accelerate development of broad-spectrum pathogen resistance in crops and contribute to global food security.

Main Text:

Pathogens and host organisms are locked in an evolutionary competition: the host immune system strives to detect attacks while pathogens evolve to escape detection. At the molecular level, host immune recognition often hinges on direct interaction between a pathogen-derived molecule and an immune protein. Pathogens can accumulate mutations that escape such interactions, with their short lifecycles and large populations giving them an advantage. Directed evolution harnesses high-throughput screening of synthetic protein sequences and effectively compresses the evolutionary timescale. Therefore, directed evolution can shift the balance in favor of the host by accelerating the development of immune proteins that recognize a wide array of pathogen-derived molecules, including those that previously evaded detection.

Directed evolution can benefit plants in a unique manner. Unlike animals with their adaptive immune systems, plants carry hundreds to thousands of genome-encoded immune receptors. Many of these receptors are activated by direct binding to effectors, pathogen-secreted molecules that facilitate infection (*1*). Developing novel immune receptors that can bind previously unrecognized effectors, therefore, can protect crops against a broader spectrum of pathogens. Increasing global temperatures, which is predicted to enhance pathogen spread and reduce plant immunity, adds urgency to development of such receptors (*2*, *3*). While gain-of-function random mutagenesis screens have led to improved immune receptors, their utility has been constrained by the limited throughput of *in planta* testing (*4–6*). Our goal was to establish a rapid, high-throughput method to explore vast synthetic sequence spaces and identify variants with broad-spectrum ligand recognition. We chose Pik1, an intracellular NOD-like receptor (NLR) in rice with a well-characterized effector binding domain (*7–9*), as our engineering target.

Rice blast, a fungal disease caused by *Magnaporthe oryzae*, leads to annual loss of rice enough to feed 60 million people (*10*, *11*). Introduction of immune receptors that protect against *M. oryzae* into crops presents a sustainable solution to this global threat. Pik1 recognizes the *M. oryzae* effector Avr-Pik through its heavy metal associated (HMA) domain, which has been co-opted from rice endogenous targets of Avr-Pik (*7*, *12*, *13*). Due to its modular nature, the Pik1 HMA domain has been engineered extensively to alter ligand binding properties while retaining Pik1 function in immunity (*14–18*). While Pik1 recognition of Avr-Pik effectively triggers disease resistance, some *M. oryzae* strains harbor Avr-Pik variants with point mutations that escape detection by all known Pik1 alleles, and some strains lack Avr-Pik altogether. 85% of sequenced rice-infecting *M. oryzae* strains that lack Avr-Pik variants harbor AvrPiz-t, a sequence divergent effector that belongs to the same family as Avr-Pik (*Magnaporthe* AVRs and ToxB-like, or MAX effector family) (*19*, *20*).

Coupling heterologous single cell expression with rounds of directed evolution (Fig. 1A), we engineered Pik1 HMA domains that bind all Avr-Pik alleles. We then further optimized these domains for affinity to AvrPiz-t, reasoning that an engineered immune receptor that binds both sets of effectors would lead to broad spectrum resistance. Our results reveal that directed evolution can yield the new-to-nature function of recognizing multiple sequence divergent ligands and indicate a new avenue for enhancing crop resistance to emerging pathogens.

Plant immune receptor-ligand interactions are recapitulated in a yeast expression platform

We first set out to test whether the Pik1 HMA domain could retain its effector interaction properties when expressed on *Saccharomyces cerevisiae*. In yeast surface display, recombinant proteins fused to a cell wall protein are displayed on the surface, allowing screening of up to ~10^9 variants in one experiment. This approach has identified proteins with desired ligand interactions, including novel receptor domains that bind pathogen-derived molecules or antibodies with antitumor or pathogen neutralizing activities (*21–23*). We expressed three well-characterized rice genome-encoded alleles of Pik1 HMA - Pikp1, Pikm1, and Pikh1 – tethered to the yeast Aga2p cell wall protein and flanked by epitope tags (Fig. 1B).

We tested whether effector binding specificity and relative affinity were recapitulated by yeast surface displayed Pik1 HMA domains (Fig. 1C). Fluorescence imaging of Myc epitope-stained yeast cells showed expression of Pik1 HMA domains on the cell surface and their interaction

with biotin-labeled effectors (Fig. 1D and Fig supp. 1A). The Pikh1 HMA domain showed strong affinity for Avr-PikD, moderate affinity for Avr-PikA, but no significant interaction with Avr-PikC and Avr-PikF variants of the effector, reflecting the effector binding properties of this allele in rice (*24*). Flow cytometry analysis of Pik1 HMA domain-expressing yeast cells recapitulated these results (Fig. 1E and Fig supp. 1B and C). These results validated that our yeast expression platform reflects the specificity and affinity of Pik1 immune receptor-ligand interactions.

Directed evolution of Pik1 HMA domain variants that bind previously unrecognized ligands

We then sought to engineer yeast displayed Pikh1 HMA domain to bind previously unrecognized effectors through directed evolution. We first subjected the HMA domain to random mutagenesis through error prone PCR. Mutagenized amplicons were introduced into yeast cells to generate a library of Pikh1 HMA domain variants. Colony dilution estimated the library to contain 2.2×10^7 variants and sequencing a subset of the library showed that the average number of amino acid mutations was 2.1 over the 78aa effector binding domain, with majority of the variants containing one to three amino acid changes (Fig supp. 2A and Table S1).

To identify Pikh1 HMA domain variants that bind the previously unrecognized effectors Avr-PikC and Avr-PikF, we performed four rounds of FACS selection (Fig. 2A). Starting with the library, we exposed yeast cells displaying Pikh1 HMA domain variants to either biotin-labeled Avr-PikC or Avr-PikF and selected those showing the highest affinity for the target effector and robust HMA domain expression. In subsequent selection rounds, variants were incubated with lower concentrations of the target effector alternating between Avr-PikC or Avr-PikF, with the goal of identifying variants that bind both effectors with high affinity (Fig. 2A). In each selection round we sorted through enough cells to oversample the population diversity by five- to ten-fold to avoid losing unique variants, selecting cells in the top 0.5-1% for target binding and HMA domain expression to carry onto the next round (Fig supp. 2B).

Following four rounds of selection, variants gained binding to both Avr-PikC and Avr-PikF at the population level regardless of the order in which they were exposed to the two effectors (Fig. 2B). Illumina sequencing of these populations showed that few variants had increased in representation over selection rounds (Table S2). Of these, variants we named A4.1 and B4.1 showed greatest enrichment relative to the starting library at the end of selection path A and selection path B, respectively (Table S3).

These variants indeed exhibited high affinity for both Avr-PikC or Avr-PikF (Fig. 2C). Flow cytometry and imaging analysis indicated that their affinities for Avr-PikC and Avr-PikF were comparable to or exceeded the binding affinity required for Pik1-induced *in planta* immune response (Fig supp. 2C and Fig supp. 1C). Importantly, A4.1 and B4.1 retained the ability to bind all effectors recognized by the wildtype Pikh1 HMA domain: Avr-PikA, Avr-PikD, and Avr-PikE (Fig supp. 2D). A4.1 and B4.1 also showed binding to a novel allele of Avr-Pik we identified in *M. oryzae* isolates TW-6-2-2-B-1 and JS-10-6-1-2 and named Avr-PikJ (Fig supp. 2D). An Avr-Pik-like effector with ~60% sequence identity to Avr-Pik, Avr-PikL2A, was identified in rice-infecting *M. oryzae* (Fig supp. 2E) (*25*). Variants A4.1 and B4.1 bound Avr-PikL2A whereas the wildtype Pikh1 HMA domain failed to do so (Fig supp. 2D).

A4.1 and B4.1 harbor five substitutions, with two of them (Q74R and A75V of the HMA domain sequence) shared by both variants (Fig. 2D). Interestingly, A75V is also present in interface 3 of the natural allele Pikm1, which is thought to contribute extensively to the interaction with Avr-Pik effectors in this allele (Fig supp. 2F) (*26*). However, the rest of the mutations that conferred broad spectrum Avr-Pik binding in A4.1 and B4.1 had not been observed in rice genome-encoded or previously engineered alleles of Pik1. Therefore, these two Pikh1 HMA domain variants gained the ability to bind all known alleles of Avr-Pik through unique, new-to-nature mutations.

Directed evolution of Pik1 HMA domain variants with broad spectrum ligand binding

Directed evolution can yield a receptor that binds allelic variants of its native ligand, but can additional rounds of selection lead to affinity for a more divergent ligand? To test this, we targeted AvrPiz-t. While Avr-Pik and AvrPiz-t both belong to the MAX family of *M. oryzae* effectors characterized by their β-sandwich structures, they exhibit no detectable sequence similarity (Fig. 3A and Fig supp. 2E). Avr-Pik and AvrPiz-t are recognized by two distinct rice genome-encoded immune receptors and interact with different sets of host proteins to suppress immunity, highlighting different evolutionary trajectories and functions of the two effectors (*12*, *19*, *27–29*). AvrPiz-t is present in 85% of rice-infecting *M. oryzae* that lack all alleles of Avr-Pik, and 193 out of 201 rice-infecting isolates with genomes available on NCBI harbor either Avr-Pik or AvrPiz-t. Its divergence from Avr-Pik, high conservation in *M. oryzae* strains that lack Avr-Pik, and role in facilitating pathogen infection rendered AvrPiz-t both a good test case and a practical target for broad spectrum *M. oryzae* recognition by an engineered receptor.

To identify Pikh1 HMA domain variants that bind AvrPiz-t in addition to Avr-PikC and Avr-PikF, we pooled variants collected after four rounds of selection through paths A and B. These variants were subjected to two subsequent rounds of selection to identify those with affinity for decreasing concentrations of AvrPiz-t (Fig. 3B). As top HMA variants identified at the end of the selection process did not exhibit strong binding to AvrPiz-t, we further diversified the post-sort 6 pool through error-prone PCR. This new library was estimated to contain 2.1×10^7 variants through colony dilution, with 4.4 mutations over the 78aa effector binding domain on average (Fig supp. 3A). Variants collected after one additional round of selection with 200nM AvrPiz-t showed enhanced affinity for AvrPiz-t at the population level (Fig. 3C and Fig supp. 3B). We evaluated Pikh1 HMA variants that were significantly enriched following sort 7 relative to their frequencies in the post-sort 6 mutagenized library (Table S3). Among these, variant 7.2 demonstrated strong binding to AvrPiz-t as well as Avr-PikC and Avr-PikF in confocal imaging and flow cytometry analyses (Fig. 3C and 3D and Fig supp. 3D). Additionally, 7.2 showed affinity for all tested alleles of Avr-Pik in confocal imaging (Fig supp. 3C).

Interestingly, the HMA domain variant with three (Q4R, K5I, and M12R) of the substitutions in 7.2 was present at low frequencies after four rounds of selection for Avr-PikC and Avr-PikF binding (Table S2). It then became the predominant variant following selection rounds 5 and 6 for AvrPiz-t binding. However, these changes alone were insufficient to confer robust affinity for Avr-PikC, Avr-PikF, and AvrPiz-t (data not shown). The additional E13K and E68K substitutions in variant 7.2 appear to account for the improved binding to all three ligands. In conclusion, iterative cycles of stringent selection and diversity enhancement through random mutagenesis yielded a HMA domain variant capable of binding all Avr-Pik alleles and AvrPiz-t.

Discussion

Our results demonstrate the first successful application of high throughput directed evolution to engineer a plant immune receptor domain that can bind previously unrecognized ligands. Lab-evolved Pikh1 HMA domain variant 7.2 displayed binding to Avr-Pik and AvrPiz-t, *M. orzyae* effectors from two sequence divergent families. An immune receptor that binds evolutionarily divergent effectors with distinct roles in pathogen infection is predicted to confer broad spectrum resistance in crops. Such a receptor will likely maintain its effectiveness barring loss of all target effector expression in the pathogen.

Multiple evolutionary paths led to expanded effector perception in our experiments. Variants A4.1, B4.1, and 7.2 each gained novel binding properties through five synergistic amino acid substitutions. A4.1 and B4.1 were obtained from different selection schemes and shared two out of their five amino acid changes. Yet both variants showed affinity for all known alleles of the effector Avr-Pik. Variant 7.2 additionally gained the ability to recognize AvrPiz-t through a yet different set of mutations. Substitutions in these engineered variants revealed residues relevant for effector recognition outside of the interfaces identified in receptor-effector crystal structures (*9*, *14*, *24*, *26*).

Plant immune receptor engineering has largely relied on grafting ligand binding domains or subsets thereof from genome-encoded variants and testing chimeric receptors in individual plants. While effective, this approach is labor intensive and requires extensive structural knowledge of the receptor-effector interface, which is not available yet for most plant immune receptors (*30*). Directed evolution in a heterologous system can expand effector recognition spectra of immune receptors at a faster pace even without prior structural knowledge. For instance, a loss-of-function random mutagenesis screen performed in yeast can quickly identify the receptor domain required for interaction with a cognate effector. A subsequent gain-of-function screen, as described here, searches for receptor variants with affinity for previously unrecognized effectors.

Enhancing effector recognition through substitutions identified through high throughput screening offers additional advantages over introducing exogenous immune receptors or replacing large receptor segments. These small changes are more amenable to current crop genome editing technologies. Introducing precise amino acid changes into a genome-encoded receptor such as Pik1 through prime editing, for example, presents a feasible path to develop and deploy improved crops (31, 32).

Functional validation in plants remains critical to confirm that these targeted substitutions preserve Pik1 immune function. Nevertheless, our results demonstrate how high throughput directed evolution can explore large synthetic sequence spaces to develop immune recognition of divergent effectors, a function that seemed initially far-fetched. We establish directed evolution as a strategy to develop broad-spectrum resistance and protect crops from fast evolving pathogens.

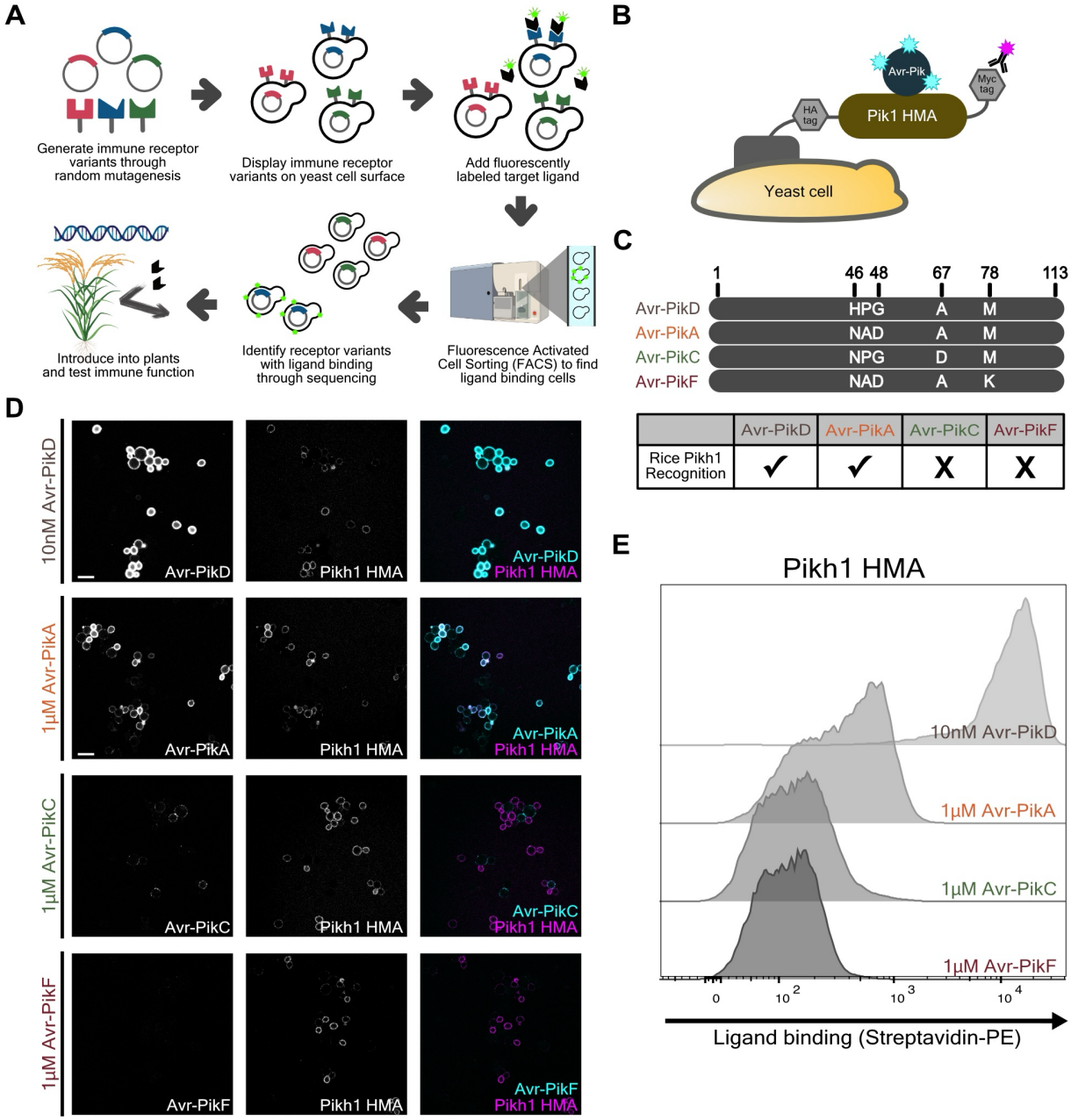
A
Generate immune receptor variants through random mutagenesis
Display immune receptor variants on yeast cell surface
Add fluorescently labeled target ligand
Fluorescence Activated Cell Sorting (FACS) to find ligand binding cells
Identify receptor variants with ligand binding through sequencing
Introduce into plants and test immune function
B
Avr-Pik
HA tag
Myc tag
Pik1 HMA
Yeast cell
C
1
46 48
67
78
113
Avr-PikD HPG A M
Avr-PikA NAD A M
Avr-PikC NPG D M
Avr-PikF NAD A K
Avr-PikD Avr-PikA Avr-PikC Avr-PikF
Rice Pikh1 Recognition ✓ ✓ X X
D
10nM Avr-PikD
1μM Avr-PikA
1μM Avr-PikC
1μM Avr-PikF
Avr-PikD
Pikh1 HMA
Avr-PikD Pikh1 HMA
Avr-PikA
Avr-PikA Pikh1 HMA
Avr-PikC
Avr-PikC Pikh1 HMA
Avr-PikF
Avr-PikF Pikh1 HMA
E
Pikh1 HMA
10nM Avr-PikD
1μM Avr-PikA
1μM Avr-PikC
1μM Avr-PikF
0
10 2
10 3
10 4
Ligand binding (Streptavidin-PE)

Fig. 1. Yeast surface-displayed rice receptor heavy metal associated (HMA) domain recapitulates its endogenous ligand binding properties. (**A**) A schematic representation of directed evolution of a plant immune receptor with desired binding properties through yeast surface display. A library of variants is expressed on single yeast cells. Variants that have gained binding to the target effector are identified through fluorescence-activated cell sorting (FACS) selection. (**B**) A schematic representation of Pikh1 yeast surface display. Pikh1 HMA domain expression and its interaction with target Avr-Pik are assessed through two-color fluorescence detection. (**C**) Allelic variants of the *M. oryzae* effector Avr-Pik. Rice receptor Pikh1 elicits immune response in the presence of Avr-PikD and Avr-PikA, but not Avr-PikC and Avr-PikF. (**D**) Immunofluorescence images of yeast cells expressing Pikh1 HMA domain. Left: biotin-labeled effector binding at specified concentrations detected through Alexa Fluor 488-conjugated streptavidin. Middle: HMA domain expression detected through anti-Myc tag binding to Alexa Fluor 568. Right: merged images of effector binding (cyan) and receptor expression (magenta). Scale bar, 10µm. (**E**) Flow cytometry quantification of the interaction between yeast surface-displayed Pikh1 HMA domain and biotin-labeled Avr-Pik variants detected through phycoerythrin-conjugated streptavidin.

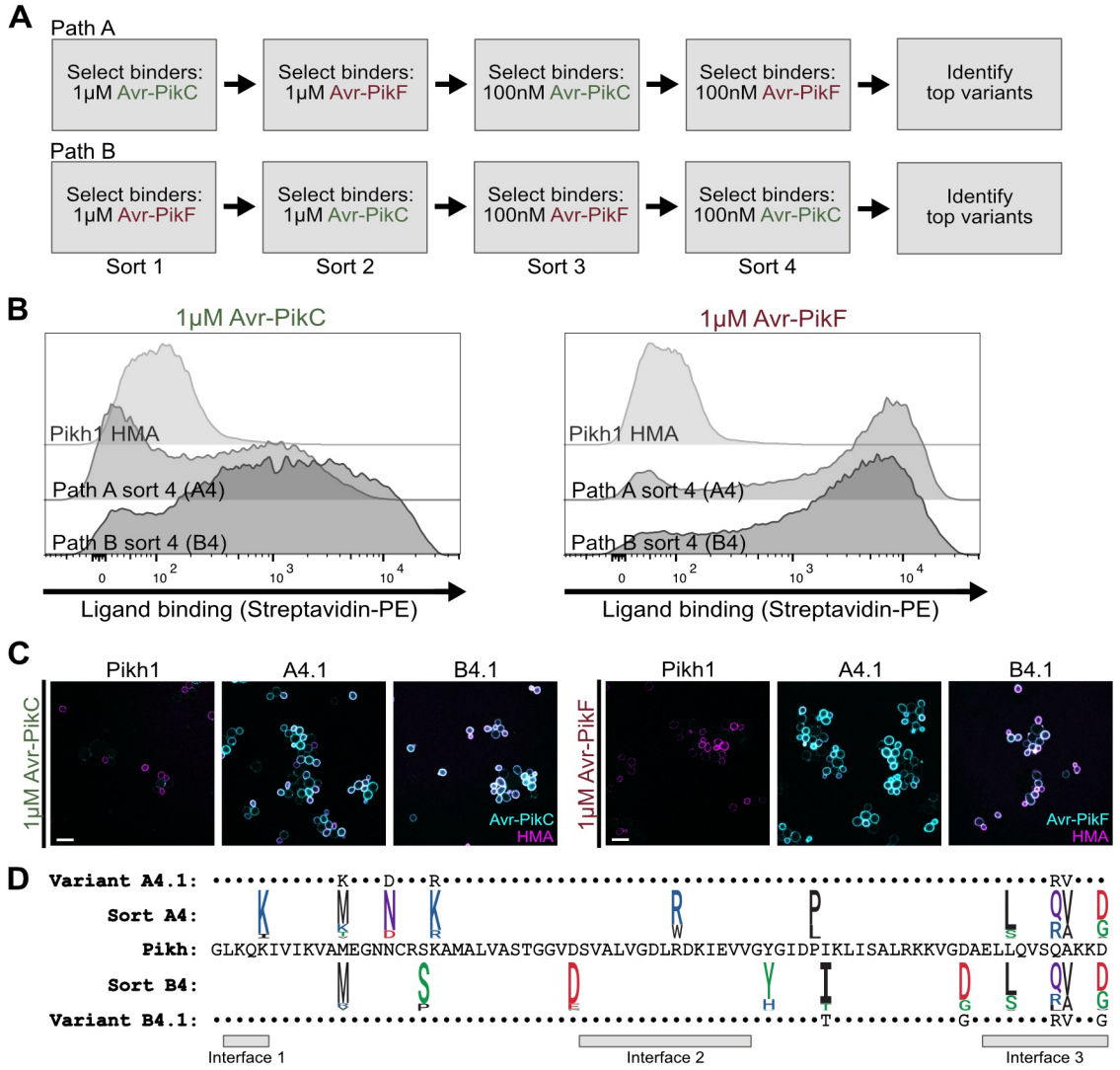

Fig. 2. Rounds of FACS selection identify Pik1 HMA domain variants with affinity for novel ligands. (**A**) Directed evolution workflow to develop binding to both Avr-PikC and Avr-PikF. Variants were selected for binding to alternating effectors at decreasing concentrations in two distinct paths. (**B**) Flow cytometry quantification of the interaction between Pikh1 HMA domains and 1μM Avr-PikC or 1μM Avr-PikF. Compared to the wildtype Pikh1 HMA domain (top), variants collected at the end of selection path A (middle) and path B (bottom) show increased affinity for Avr-PikC (left) and Avr-PikF (right). (**C**) Immunofluorescence images of yeast cells expressing Pikh1 HMA domains in the presence of 1μM Avr-PikC (left) or 1μM Avr-PikF (right). Merged images show biotin-labeled effector binding detected through Alexa Fluor 488-conjugated streptavidin (cyan) and receptor expression detected through anti-Myc tag binding to Alexa Fluor 568 (magenta) of the wildtype Pikh1, A4.1, and B4.1 HMA domains. Scale bar, 10μm. (**D**) Amino acid sequences of the wildtype Pikh1 HMA domain and variants A4.1 and B4.1 enriched at the end of selection paths A and B, respectively. Sequence logos depict the 10 most frequently mutated residues in variants after sort 4 of each selection path (red: acidic, blue: basic, black: hydrophobic, purple: neutral, green: polar). Interfaces known to interact with Avr-Pik are indicated below (*9*, *16*).

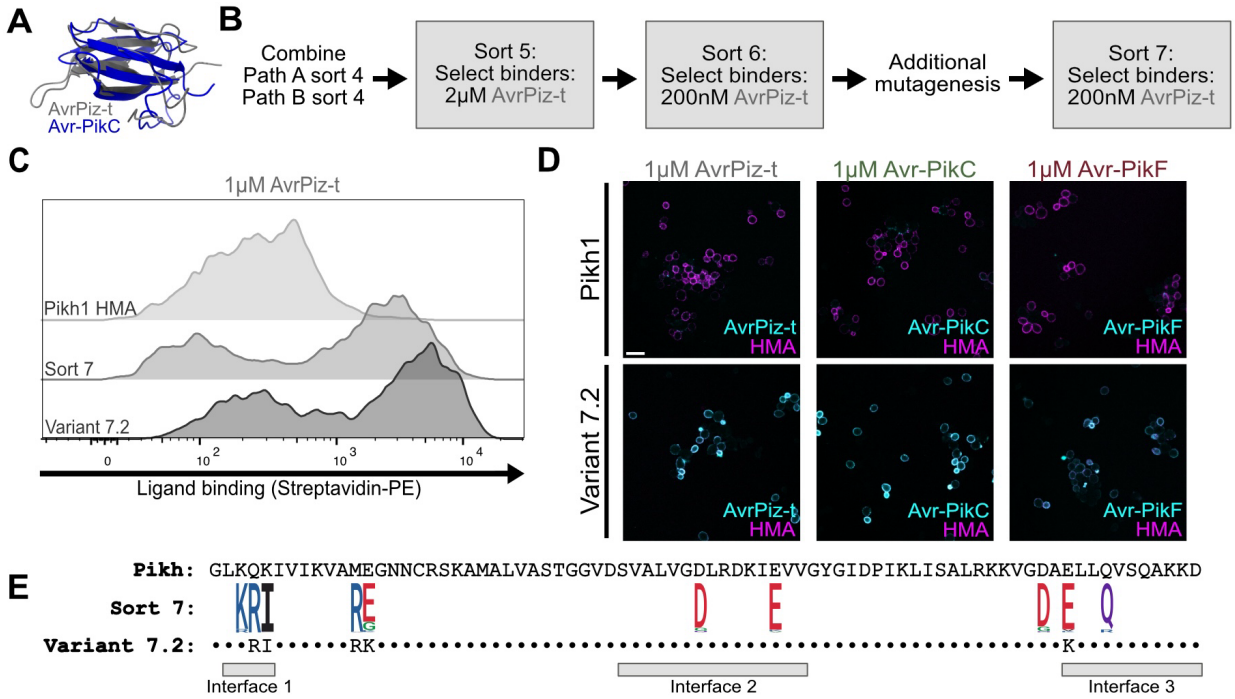

Fig. 3. Additional FACS selection identifies Pik1 HMA domain variants with affinity for a structurally divergent ligand. (**A**) Overlay of crystal structures of the MAX effectors Avr-PikC (blue, PDB: 7A8X) and AvrPiz-t (grey, PDB: 2LW6). (**B**) Directed evolution workflow to develop binding to AvrPiz-t. Variants were selected for binding to AvrPiz-t at decreasing concentrations, with an additional round of random mutagenesis to restore library diversity. (**C**) Flow cytometry quantification of the interaction between Pikh1 HMA domains and 1μM AvrPiz-t. Compared to the wildtype Pikh1 HMA domain (top), variants collected at the end of sort 7 (middle) and selected variant 7.2 (bottom) show increased affinity for AvrPiz-t. (**D**) Immunofluorescence images of yeast cells expressing Pikh1 HMA domains in the presence of 1μM AvrPiz-t (left), 1μM Avr-PikC (middle), or 1μM Avr-PikF (right). Merged images show biotin-labeled effector binding detected through Alexa Fluor 488-conjugated streptavidin (cyan) and receptor expression detected through anti-Myc tag binding to Alexa Fluor 568 (magenta) of the Pikh1 wildtype (top) and variant 7.2 (bottom) HMA domains. Scale bar, 10μm. (**E**) Amino acid sequences of the wildtype Pikh1 HMA domain and variant 7.2 are shown. Sequence logo depicts the 10 most frequently mutated residues in variants after sort 7 (red: acidic, blue: basic, black: hydrophobic, purple: neutral, green: polar). Interfaces known to interact with Avr-Pik are indicated below (*9*, *16*).

References

1. M. F. Ercoli, D. D. Luu, E. Y. Rim, A. Shigenaga, A. Teixeira de Araujo, M. Chern, R. Jain, R. Ruan, A. Joe, V. Stewart, P. Ronald, Plant immunity: Rice XA21-mediated resistance to bacterial infection. *Proc. Natl. Acad. Sci. U.S.A.* **119**, e2121568119 (2022).

2. N. A. Eckardt, E. A. Ainsworth, R. N. Bahuguna, M. R. Broadley, W. Busch, N. C. Carpita, G. Castrillo, J. Chory, L. R. DeHaan, C. M. Duarte, A. Henry, S. V. K. Jagadish, J. A. Langdale, A. D. B. Leakey, J. C. Liao, K.-J. Lu, M. C. McCann, J. K. McKay, D. A. Odeny, E. Jorge De Oliveira, J. D. Platten, I. Rabbi, E. Y. Rim, P. C. Ronald, D. E. Salt, A. M. Shigenaga, E. Wang, M. Wolfe, X. Zhang, Climate change challenges, plant science solutions. *The Plant Cell* **35**, 24–66 (2023).

3. A. C. Velásquez, C. D. M. Castroverde, S. Y. He, Plant–Pathogen Warfare under Changing Climate Conditions. *Current Biology* **28**, R619–R634 (2018).

4. M. E. Segretin, M. Pais, M. Franceschetti, A. Chaparro-Garcia, J. I. B. Bos, M. J. Banfield, S. Kamoun, Single Amino Acid Mutations in the Potato Immune Receptor R3a Expand Response to *Phytophthora* Effectors. *MPMI* **27**, 624–637 (2014).

5. G. Farnham, D. C. Baulcombe, Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene from potato. *Proceedings of the National Academy of Sciences* **103**, 18828–18833 (2006).

6. C. J. Harris, E. J. Slootweg, A. Goverse, D. C. Baulcombe, Stepwise artificial evolution of a plant disease resistance gene. *Proceedings of the National Academy of Sciences* **110**, 21189–21194 (2013).

7. I. Ashikawa, N. Hayashi, H. Yamane, H. Kanamori, J. Wu, T. Matsumoto, K. Ono, M. Yano, Two Adjacent Nucleotide-Binding Site–Leucine-Rich Repeat Class Genes Are Required to Confer *Pikm* -Specific Rice Blast Resistance. *Genetics* **180**, 2267–2276 (2008).

8. K. Yoshida, H. Saitoh, S. Fujisawa, H. Kanzaki, H. Matsumura, K. Yoshida, Y. Tosa, I. Chuma, Y. Takano, J. Win, S. Kamoun, R. Terauchi, Association Genetics Reveals Three Novel Avirulence Genes from the Rice Blast Fungal Pathogen *Magnaporthe oryzae*. *The Plant Cell* **21**, 1573–1591 (2009).

9. A. Maqbool, H. Saitoh, M. Franceschetti, C. Stevenson, A. Uemura, H. Kanzaki, S. Kamoun, R. Terauchi, M. Banfield, Structural basis of pathogen recognition by an integrated HMA domain in a plant NLR immune receptor. *eLife* **4**, e08709 (2015).

10. L. Nalley, F. Tsiboe, A. Durand-Morat, A. Shew, G. Thoma, Economic and Environmental Impact of Rice Blast Pathogen (Magnaporthe oryzae) Alleviation in the United States. *PLoS ONE* **11**, e0167295 (2016).

11. E. Pennisi, Armed and Dangerous. *Science* **327**, 804–805 (2010).

12. K. Oikawa, K. Fujisaki, M. Shimizu, T. Takeda, H. Saitoh, A. Hirabuchi, Y. Hiraka, A. Białas, T. Langner, R. Kellner, T. O. Bozkurt, S. Cesari, T. Kroj, J. H. R. Maidment, M. J.

Banfield, S. Kamoun, R. Terauchi, "The blast pathogen effector AVR-Pik binds and stabilizes rice heavy metal-associated (HMA) proteins to co-opt their function in immunity" (preprint, Plant Biology, 2020); https://doi.org/10.1101/2020.12.01.406389.

13. A. Białas, T. Langner, A. Harant, M. P. Contreras, C. E. Stevenson, D. M. Lawson, J. Sklenar, R. Kellner, M. J. Moscou, R. Terauchi, M. J. Banfield, S. Kamoun, Two NLR immune receptors acquired high-affinity binding to a fungal effector through convergent evolution of their integrated domain. *eLife* **10**, e66961 (2021).

14. J. H. Maidment, M. Shimizu, A. R. Bentham, S. Vera, M. Franceschetti, A. Longya, C. E. Stevenson, J. C. De La Concepcion, A. Białas, S. Kamoun, R. Terauchi, M. J. Banfield, Effector target-guided engineering of an integrated domain expands the disease resistance profile of a rice NLR immune receptor. *eLife* **12**, e81123 (2023).

15. J. C. De la Concepcion, M. Franceschetti, D. MacLean, R. Terauchi, S. Kamoun, M. J. Banfield, Protein engineering expands the effector recognition profile of a rice NLR immune receptor. *eLife* **8**, e47713 (2019).

16. J. Kourelis, C. Marchal, A. Posbeyikian, A. Harant, S. Kamoun, NLR immune receptor–nanobody fusions confer plant disease resistance. *Science* **379**, 934–939 (2023).

17. R. Zdrzałek, Y. Xi, T. Langner, A. R. Bentham, Y. Petit-Houdenot, J. C. De La Concepcion, A. Harant, M. Shimizu, V. Were, N. J. Talbot, R. Terauchi, S. Kamoun, M. J. Banfield, Bioengineering a plant NLR immune receptor with a robust binding interface toward a conserved fungal pathogen effector. *Proc. Natl. Acad. Sci. U.S.A.* **121**, e2402872121 (2024).

18. S. Cesari, Y. Xi, N. Declerck, V. Chalvon, L. Mammri, M. Pugnière, C. Henriquet, K. De Guillen, V. Chochois, A. Padilla, T. Kroj, New recognition specificity in a plant immune receptor by molecular engineering of its integrated domain. *Nat Commun* **13**, 1524 (2022).

19. K. de Guillen, D. Ortiz-Vallejo, J. Gracy, E. Fournier, T. Kroj, A. Padilla, Structure Analysis Uncovers a Highly Diverse but Structurally Conserved Effector Family in Phytopathogenic Fungi. *PLoS Pathog* **11**, e1005228 (2015).

20. W. Li, B. Wang, J. Wu, G. Lu, Y. Hu, X. Zhang, Z. Zhang, Q. Zhao, Q. Feng, H. Zhang, Z. Wang, G. Wang, B. Han, Z. Wang, B. Zhou, The *Magnaporthe oryzae* Avirulence Gene *AvrPiz-t* Encodes a Predicted Secreted Protein That Triggers the Immunity in Rice Mediated by the Blast Resistance Gene *Piz-t*. *MPMI* **22**, 411–420 (2009).

21. N. Mehta, S. Maddineni, R. L. Kelly, R. B. Lee, S. A. Hunter, J. L. Silberstein, R. A. Parra Sperberg, C. L. Miller, A. Rabe, L. Labanieh, J. R. Cochran, An engineered antibody binds a distinct epitope and is a potent inhibitor of murine and human VISTA. *Sci Rep* **10**, 15171 (2020).

22. J. W. Kim, C. P. Marquez, K. Kostyrko, A. L. Koehne, K. Marini, D. R. Simpson, A. G. Lee, S. G. Leung, L. C. Sayles, J. Shrager, I. Ferrer, L. Paz-Ares, M. H. Gephart, S. Vicent, J. R. Cochran, E. A. Sweet-Cordero, Antitumor activity of an engineered decoy receptor

targeting CLCF1–CNTFR signaling in lung adenocarcinoma. *Nat Med* **25**, 1783–1795 (2019).

23. A. Glasgow, J. Glasgow, D. Limonta, P. Solomon, I. Lui, Y. Zhang, M. A. Nix, N. J. Rettko, S. Zha, R. Yamin, K. Kao, O. S. Rosenberg, J. V. Ravetch, A. P. Wiita, K. K. Leung, S. A. Lim, X. X. Zhou, T. C. Hobman, T. Kortemme, J. A. Wells, Engineered ACE2 receptor traps potently neutralize SARS-CoV-2. *Proc Natl Acad Sci USA*, 202016093 (2020).

24. J. C. De la Concepcion, J. H. R. Maidment, A. Longya, G. Xiao, M. Franceschetti, M. J. Banfield, The allelic rice immune receptor Pikh confers extended resistance to strains of the blast fungus through a single polymorphism in the effector binding interface. *PLoS Pathog* **17**, e1009368 (2021).

25. A. R. Bentham, Y. Petit-Houdenot, J. Win, I. Chuma, R. Terauchi, M. J. Banfield, S. Kamoun, T. Langner, A single amino acid polymorphism in a conserved effector of the multihost blast fungus pathogen expands host-target binding spectrum. *PLoS Pathog* **17**, e1009957 (2021).

26. J. C. De la Concepcion, M. Franceschetti, A. Maqbool, H. Saitoh, R. Terauchi, S. Kamoun, M. J. Banfield, Polymorphic residues in rice NLRs expand binding and response to effectors of the blast pathogen. *Nat Plants* **4**, 576–585 (2018).

27. Z.-M. Zhang, X. Zhang, Z.-R. Zhou, H.-Y. Hu, M. Liu, B. Zhou, J. Zhou, Solution structure of the Magnaporthe oryzae avirulence protein AvrPiz-t. *J Biomol NMR* **55**, 219–223 (2013).

28. B. Zhou, S. Qu, G. Liu, M. Dolan, H. Sakai, G. Lu, M. Bellizzi, G.-L. Wang, The Eight Amino-Acid Differences Within Three Leucine-Rich Repeats Between Pi2 and Piz-t Resistance Proteins Determine the Resistance Specificity to *Magnaporthe grisea*. *MPMI* **19**, 1216–1228 (2006).

29. C.-H. Park, S. Chen, G. Shirsekar, B. Zhou, C. H. Khang, P. Songkumarn, A. J. Afzal, Y. Ning, R. Wang, M. Bellizzi, B. Valent, G.-L. Wang, The *Magnaporthe oryzae* Effector AvrPiz-t Targets the RING E3 Ubiquitin Ligase APIP6 to Suppress Pathogen-Associated Molecular Pattern–Triggered Immunity in Rice. *Plant Cell* **24**, 4748–4762 (2012).

30. A. R. Bentham, J. C. De La Concepcion, N. Mukhi, R. Zdrzałek, M. Draeger, D. Gorenkin, R. K. Hughes, M. J. Banfield, A molecular roadmap to the plant immune system. *Journal of Biological Chemistry* **295**, 14916–14935 (2020).

Acknowledgments: We thank A. Bentham and M. Banfield for sharing pOPIN-GG vectors and for advice on protein expression and purification. This project was supported by the University of California Davis Flow Cytometry Shared Resource Laboratory with technical assistance from Bridget McLaughlin, Jonathan Van Dyke and Ashley Karajeh.

Funding:

Life Sciences Research Foundation – Simons Foundation (EYR)

National Institutes of Health MIRA 1R35GM148173 (PCR)

National Science Foundation IOS-2139987 (SPD-K)

National Institutes of Health grant P30 CA093373 (Flow Cytometry Shared Resource)

National Institutes of Health NCRR C06-RR1208 (Flow Cytometry Shared Resource)

National Institutes of Health S10 OD018223 (Flow Cytometry Shared Resource)

National Institutes of Health S10 RR 026825 (Flow Cytometry Shared Resource)

James B. Pendleton Charitable Trust (Flow Cytometry Shared Resource)

Partially supported by the Joint BioEnergy Institute, U.S. Department of Energy, Office of Science, Biological and Environmental Research Program under Award Number DE-AC02-05CH11231 (PCR)

Competing interests: E.Y.R. and P.C.R. are inventors on a provisional patent (No. 63/692,024) related to this work.

Supplementary Materials

Materials and Methods

Figs. S1 to S3

Tables S1 to S4