Title: NLR immune receptor-nanobody fusions confer plant disease resistance

Authors: Jiorgos Kourelis[†], Clemence Marchal[†], Andres Posbeyikian, Adeline Harant, Sophien Kamoun[*]

Affiliations:

The Sainsbury Laboratory, University of East Anglia, Norwich Research Park, NR4 7UH, Norwich, UK

[†]These authors contributed equally to this work

[*]Correspondence: sophien.kamoun@tsl.ac.uk

Abstract: Plant pathogens cause recurrent epidemics, threatening crop yield and global food security. Efforts to retool the plant immune system have been limited to modifying natural components and can be nullified by the emergence of new pathogen strains. Made-to-order synthetic plant immune receptors provide an opportunity to tailor resistance to pathogen genotypes present in the field. Here we show that plant immune receptors can be used as scaffolds for nanobody (single-domain antibody fragment) fusions that bind fluorescent proteins (FPs). These fusions trigger immune responses in the presence of the corresponding FP, and confer resistance against plant viruses expressing FPs. Since nanobodies can be raised against most molecules, immune receptor-nanobody fusions have the potential to generate resistance against plant pathogens and pests delivering effectors inside host cells.

One-Sentence Summary: Plant immune receptor-nanobody fusions enable made-to-order disease resistance genes.

Introduction

Plants lack an adaptive immune system and rely on innate immune receptors to detect invading pathogens. Efforts to retool the plant immune system to design new-to-nature biochemical activities have been largelylimited to modification of natural components, for instance through receptor mutagenesis or domain shuffling (*1–8*). While these approaches have yielded promising results, they often target a specific pathogen isolate and thus lack plasticity and adaptability to a wider range of pathogens and pests. In addition, plant pathogens are notorious for rapidly evolving virulent races that can nullify new resistance specificities. Thus, there is a need for an adaptative system where new resistance can be bioengineered as required to target the pathogen genotypes associated with plant disease outbreaks.

One class of immune proteins that could be optimal templates for receptor bioengineering are the subset of intracellular NOD-like/nucleotide-binding leucine-rich repeat immune receptors (NLRs) that carry unconventional integrated domains (IDs) (*9–12*). These IDs are generally thought to mediate pathogen effector detection, either by directly binding to effectors or by acting as a substrate for their enzymatic activity. This activity is subsequently translated into an immune response (*13–17*). Often these ID-containing NLRs (NLR-IDs) are genetically linked to conventional NLRs that are required for immune activation following effector detection (*9*, *18*). Pik-1 and Pik-2 are such an NLR receptor pair from rice carrying an N-terminal coiled-coil (CC) domain (*19*). Pik-1 carries an integrated heavy metal associated (HMA) domain between its CC and the central NB-ARC (nucleotide-binding domain shared with APAF-1, various R-proteins and CED-4) domains that directly binds AVR-Pik effector proteins (avirulence factors) secreted by the blast fungus, *Magnaporthe oryzae* (*13*, *16*, *20–*

24). AVR-Pik binding to Pik-1 HMA results in Pik-2-dependent immunity (*19*, *16*). The integrated HMA domain of Pik-1 can be mutated or swapped for similar HMA domains to confer recognition of different AVR-Pik alleles (*5*, *25*, *26*). The Pik-1/Pik-2 NLR gene pair has a variety of alleles (such as Pikp, Pikm, Pikh, Piks, and Pik*) that provide immunity to blast isolates expressing different AVR-Pik variants (*27*).

What would be the ultimate integrated domain for engineering made-to-order plant immune receptors? Given that animal adaptive immunity has the capacity to generate antibodies against virtually any antigen it is exposed to, we reasoned that harnessing antibodies for plant immunity would potentially enable building receptors that respond to a wide range of plant pathogen molecules. We focused on the minimal antigen-binding fragment of single-domain heavy chain antibodies (known as VHHs or nanobodies) of camelid mammals (*28–31*), because they are small, soluble, 10-15 kDa domains, which tend to correctly fold intracellularly and have many useful properties for biotechnological applications. To test our idea, we generated orthogonal Pik-1 sensors in which the integrated HMA domain is swapped with nanobodies that bind either GFP or mCherry (*32–34*) **(Fig. 1A-B**, **Table S1).** We hypothesized that the engineered versions of Pik-1 would trigger immunity in the presence of GFP or mCherry.

Results

Pikobodies are functional NLR nanobody fusions

Mutations in the Pik-1 HMA domain often lead to autoimmune activities in the absence of a ligand, and like the immune signalling in response to effector recognition this activity is dependent on the presence of Pik-2 (*23*, *25*, *26*, *35*). Hence, we first tested whether the Pikm-1-nanobody fusions induce autoimmunity in the presence of Pik-2. Of the 11 tested Pikm-1-nanobody fusions, six did not exhibit autoimmunity when expressed with Pikm-2 in leaves of

the model plant *Nicotiana benthamiana* (**Fig. S1, Table S2**), indicating that they can be used for follow-up gain-of-function assays. Next, we co-expressed ten Pikm-1 nanobody fusions with GFP or mCherry. Among these, four produced a hypersensitive cell death response (HR, immune response readout) specifically when expressed with their matching fluorescent proteins (FPs) (**Fig. 1C**, **Fig. S1**, **Table S2,** Enhancer, LaG-16, LaM-4, and LaM-8). The response levels were similar to those obtained with a natural combination of Pikm and a blast fungus effector (**Fig. 1C**, **Fig. S1**). Additionally, a further three fusions which displayed weak autoimmunity gave a stronger HR only when combined with their matching fluorescent proteins (**Fig. 1C**, **Fig. S1**, LaG-24, LaM-2, and LaM-6). This indicates that the Pikm-1-nanobody fusions are functional and can be endowed with new-to-nature activities. We coined the term Pikobody for the combination of Pikm-2 with the engineered Pikm-1^{Nanobody} fusions (**Fig. 1**).

We reasoned that nanobody aggregation or misfolding upon intracellular expression (*32*) could explain the observed autoactivity (**Fig. 1C, Fig. S1**). We introduced previously described stabilizing nanobody mutations (*32*) in LaG-24, LaM-2, LaM-3, and LaM-6 and found that they abolished Pikobody autoactivity (**Fig. S2**). Three of these Pikobodies carrying the stabilized mutants of LaG-24, LaM-3, and LaM-6 retained the capacity to trigger HR in the presence of the matching FP, while LaM-2 did not (**Fig. S2**). This shows that Pikobody autoactivity can be abolished by engineering the core structural features of the nanobodies.

Given that Enhancer and LaM-4 are widely used nanobodies recognising GFP and mCherry, respectively, we selected Pikobody^{Enhancer} (consisting of Pikm-2 together with the Pikm-1^{Enhancer} nanobody fusion) and Pikobody^{LaM-4} (consisting of Pikm-2 together with the Pikm-1^{LaM-4} nanobody fusion), to further confirm our results. Some pathogen effectors may not be recognized by Pikobodies, for example by having a subcellular localization which precludes

recognition. We noted that Pikobody^{Enhancer} and Pikobody^{LaM-4} specifically responded to three pathogen effectors only when they were tagged with the matching GFP/EGFP or mCherry/mRFP1 (**Fig. S3, Table S2**). This further confirmed that the Pikobodies are functional FP sensors that detect FPs even when they are fused to pathogen effector proteins.

We investigated the extent to which Pikobodies function through similar mechanisms as the wild-type Pik pair (*36*) and other CC- NLRs. The conserved P-loop motif within the NB-ARC domain of CC-NLRs is required for the ADP/ATP switch that enables oligomerization into resistosome complexes (*37*, *38*). Pikobody^{K217R,Enhancer} and Pikobody$^{K217R,LaM-4}$ with a P-loop dead mutation in Pikm-2 (Pikm-2^{K217R}) failed to produce a HR to their corresponding FP even though the Pikm-1 and Pikm-2 proteins accumulated to similar levels as the wild-type immune receptors (**Fig. S4, Table S2**). We conclude that the P-loop motif of Pikm-2 is required for Pikobody activity, and that the Pikobody system probably functions through the established mechanistic model of NLRs (*39*).

Transient Pikobody expression confers immunity to Potato Virus X

Can Pikobodies produce a functional immune response that is effective against a pathogen? We used recombinant *Potato virus X* (PVX) (*40*) expressing either GFP or mCherry to assay the ability of Pikobodies to reduce viral load (**Table S1**). These PVX variants express FPs from a duplicated coat protein sub-promoter in the virus genome. We used fluorescence intensity and immunodetection of GFP/mCherry accumulation as proxy for viral load in leaf samples (**Fig. 2, Table S3**). Both Pikobody^{Enhancer} and Pikobody^{LaM-4} specifically reduced fluorescence intensity of PVX expressed GFP or mCherry, respectively, to an extent comparable to that of Rx, an NLR known to confer immunity against PVX (*41*) (**Fig. 2A**-**B**). This reduction of fluorescence intensity correlates with reduced accumulation of virus expressed GFP or mCherry as compared to the empty vector control or wild-type Pikm (**Fig. 2C**-**D**). We did,

however, observe a faint signal corresponding to GFP or mCherry in the samples with PVX-GFP or PVX-mCherry and Pikobody^{Enhancer} or Pikobody^{LaM-4}, respectively, as compared to no detectable FP bands in the samples with Rx (**Fig. 2C-D**).

To independently confirm these results, we tested two additional PVX variants expressing GFP from different virus genome locations (*42*, *43*) (**Table S1**, **Table S3 Fig. S5**). Pikobody^{Enhancer}, but not Pikobody^{LaM-4} reduced GFP fluorescence intensity and protein accumulation when challenged with a PVX variant with the GFP sequence inserted between the triple gene block and coat protein in the virus genome (**Fig. S5A-B**). Furthermore, we observed reduced accumulation of virus expressed GFP in the presence of Pikobody^{Enhancer} when challenged with a PVX variant carrying an in-frame N-terminal GFP fusion to the viral coat protein separated by a foot-and-mouth disease virus 2A self-cleaving peptide, despite consistently not seeing a significant change in fluorescence intensity in the presence of Pikobody^{Enhancer} (**Fig. S5C-D**). GFP fluorescence is known to be enhanced in the presence of certain nanobodies (*33*), and perhaps the fluorescent properties of the population of GFP which remain fused to coat protein are enhanced under these conditions (**Fig. S5D**) (*43*). Additionally, at late stages of infection Pikobody^{Enhancer}, but not Pikobody^{LaM-4}, caused a visible hypersensitive cell death with both variants of PVX-GFP (**Fig. S5E-F**).

We further compared the virus resistance capacity of Pikobody^{Enhancer} to the disease resistance protein Rx using PVX variants that contain mutations in the coat protein that evade Rx-mediated immunity (*44*). Unlike Rx, Pikobody^{Enhancer} conferred resistance against the Rx-evading variant PVX-GFP::CP^{T122K,M128R} (**Fig. S6, Table S3**). We conclude that Pikobody^{Enhancer} can provide resistance against multiple PVX-GFP variants, including a variant that evades Rx.

Pikobody stacking results in additive recognition capacities

The addition of more than one immune receptor in a plant variety—a plant breeding strategy known as *R* gene stacking— can maximize resistance durability in the field by delaying the emergence of virulent pathogen races (*45–47*). However, co-expression of plant immune receptors can lead to autoimmunity (*48*, *49*) or suppression of recognition (*50*). We investigated whether Pikobodies with different FP specificities are compatible with each other **(Fig. 3).** We first determined that co-expression of different Pikobodies does not result in autoimmunity nor affect Pikobody accumulation (**Fig. S7, Table S2**). Co-expression of Pikobody^{Enhancer} or Pikobody^{LaM-4} and the wild-type Pikm pair triggered the HR only in presence of the matching FP, whereas co-expression of Pikobody^{Enhancer} and Pikobody^{LaM-4} produced the HR in the presence of both GFP and mCherry **(Fig. 3A**-**B**). Similarly, co-expression of Pikobody^{Enhancer} and Pikobody^{LaM-4} markedly reduced fluorescence intensity and protein levels of both GFP and mCherry produced by PVX-FPs (**Fig. 3C**-**F**). At late stages of infection, the combination of Pikobody^{Enhancer} and Pikobody^{LaM-4} also resulted in hypersensitive cell death in response to either PVX-GFP or PVX-mCherry (**Fig. S8**). We conclude that Pikobody stacking can expand the recognition and response profile of these immune receptors without necessarily resulting in autoimmunity.

Transgenic plant expressing Pikobodies are resistant to Potato Virus X

We challenged our findings that Pikobodies confer virus resistance using stable *N. benthamiana* transgenic lines expressing Pikobody^{Enhancer} (**Fig. 4**). Among four lines transformed with Pikobody^{Enhancer}, three specifically respond to GFP, accumulate Pikm-2 and Pikm-1^{Enhancer} proteins, and reduce virus load of PVX-GFP, but not PVX-mCherry, as estimated by accumulation of FPs and PVX coat protein (**Fig. S9**, **Table S4**, **Table S5**, line #1, #9, #10). A fourth transgenic that does not respond to GFP, and only accumulates Pikm-2 protein and not Pikm-1^{Enhancer} protein served as a negative control (**Fig. 4**, **Fig. S10**, line #4). The level of PVX resistance in one of the Pikobody^{Enhancer} transgenic lines (#9) was similar to Rx, with no

detectable levels of GFP and coat protein (**Fig. 4**, **Fig. S10)**. Importantly, the Pikobody^{Enhancer} transgenic lines conferred resistance to PVX regardless of the agroinfection method used to deliver the virus (leaf infiltration and wounding inoculation) (**Fig. 4**, **Fig. S10**, **Fig. S11**). We conclude that transgenic Pikobody lines can confer specific resistance to PVX to a similar extent as the natural resistance gene Rx.

Discussion and conclusion

We built upon our growing understanding of the evolution and function of the Pik pair of NLRs (*5, 21–23, 36*) to use Pik-1 as a chassis for VHH nanobody fusions to bioengineer functional disease resistance genes with new-to-nature functionalities. This strategy for bioengineering synthetic immune receptors contrasts with earlier approaches, which were based on the modification of endogenous sequences and domains. The Pikobody system provides a method to functionally transfer components of the metazoan immune system to plants. Given that Pikobodies rely on NLR-mediated immunity, this system shares the same limitations as other approaches leveraging this immune receptor family. For example, pathogen proteins will need to be translocated inside the plant cell during the right phase of pathogen infection to be recognized by Pikobodies. Nevertheless, given that nanobodies can be readily generated to bind virtually any antigen, Pikobodies have the potential to produce made-to-order resistance genes against any pathogen or pest that delivers effectors inside host plant cells (**Fig. S12**).

References and notes

1. S. H. Kim, D. Qi, T. Ashfield, M. Helm, R. W. Innes, Using decoys to expand the recognition specificity of a plant disease resistance protein. *Science*. **351**, 684–687 (2016).

2. S. E. Pottinger, A. Bak, A. Margets, M. Helm, L. Tang, C. Casteel, R. W. Innes, Optimizing the PBS1 decoy system to confer resistance to potyvirus infection in *Arabidopsis* and soybean. *Mol. Plant. Microbe Interact.* **33**, 932–944 (2020).

3. 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. *Mol. Plant. Microbe Interact.* **27**, 624–637 (2014).

4. A. Giannakopoulou, J. F. C. Steele, M. E. Segretin, T. O. Bozkurt, J. Zhou, S. Robatzek, M. J. Banfield, M. Pais, S. Kamoun, Tomato I2 immune receptor can be engineered to confer partial resistance to the oomycete *Phytophthora infestans* in addition to the fungus *Fusarium oxysporum*. *Mol. Plant. Microbe Interact.* **28**, 1316–1329 (2015).

5. 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).

6. S. Césari, 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).

7. S. Wang, W. Huang, Z. Duxbury, S. A. Hogenhout, J. D. G. Jones, Novel effector recognition capacity engineered into a paired NLR complex (2021), , doi:10.1101/2021.09.06.459143.

8. J. L. Dangl, D. M. Horvath, B. J. Staskawicz, Pivoting the plant immune system from dissection to deployment. *Science*. **341**, 746–751 (2013).

9. S. Césari, M. Bernoux, P. Moncuquet, T. Kroj, P. Dodds, A novel conserved mechanism for plant NLR protein pairs: the 'integrated decoy' hypothesis. *Front. Plant Sci.* **5** (2014), doi:10.3389/fpls.2014.00606.

10. C.-H. Wu, K. V. Krasileva, M. J. Banfield, R. Terauchi, S. Kamoun, The "sensor domains" of plant NLR proteins: more than decoys? *Plant-Microbe Interact.* **6**, 134 (2015).

11. T. Kroj, E. Chanclud, C. Michel-Romiti, X. Grand, J.-B. Morel, Integration of decoy domains derived from protein targets of pathogen effectors into plant immune receptors is widespread. *New Phytol.* **210**, 618–626 (2016).

12. P. F. Sarris, V. Cevik, G. Dagdas, J. D. G. Jones, K. V. Krasileva, Comparative analysis of plant immune receptor architectures uncovers host proteins likely targeted by pathogens. *BMC Biol.* **14**, 8 (2016).

13. S. Césari, G. Thilliez, C. Ribot, V. Chalvon, C. Michel, A. Jauneau, S. Rivas, L. Alaux, H. Kanzaki, Y. Okuyama, J.-B. Morel, E. Fournier, D. Tharreau, R. Terauchi, T. Kroj, The rice resistance protein pair RGA4/RGA5 recognizes the *Magnaporthe oryzae* effectors AVR-Pia and AVR1-CO39 by direct binding. *Plant Cell*. **25**, 1463–1481 (2013).

14. P. F. Sarris, Z. Duxbury, S. U. Huh, Y. Ma, C. Segonzac, J. Sklenar, P. Derbyshire, V. Cevik, G. Rallapalli, S. B. Saucet, L. Wirthmueller, F. L. H. Menke, K. H. Sohn, J. D. G. Jones, A plant immune receptor detects pathogen effectors that target WRKY transcription factors. *Cell*. **161**, 1089–1100 (2015).

15. C. Le Roux, G. Huet, A. Jauneau, L. Camborde, D. Trémousaygue, A. Kraut, B. Zhou, M. Levaillant, H. Adachi, H. Yoshioka, S. Raffaele, R. Berthomé, Y. Couté, J. E. Parker, L. Deslandes, A receptor pair with an integrated decoy converts pathogen disabling of transcription factors to immunity. *Cell*. **161**, 1074–1088 (2015).

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

17. L. Guo, S. Césari, K. de Guillen, V. Chalvon, L. Mammri, M. Ma, I. Meusnier, F. Bonnot, A. Padilla, Y.-L. Peng, J. Liu, T. Kroj, Specific recognition of two MAX effectors by integrated HMA domains in plant immune receptors involves distinct binding surfaces. *Proc. Natl. Acad. Sci.* **115**, 11637–11642 (2018).

18. T. K. Eitas, J. L. Dangl, NB-LRR proteins: pairs, pieces, perception, partners, and pathways. *Curr. Opin. Plant Biol.* **13**, 472–477 (2010).

19. 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).

20. H. Kanzaki, K. Yoshida, H. Saitoh, K. Fujisaki, A. Hirabuchi, L. Alaux, E. Fournier, D. Tharreau, R. Terauchi, Arms race co-evolution of *Magnaporthe oryzae AVR-Pik* and rice *Pik* genes driven by their physical interactions. *Plant J.* **72**, 894–907 (2012).

21. 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 (2018).

22. 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).

23. 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).

24. A. Białas, E. K. Zess, J. C. De la Concepcion, M. Franceschetti, H. G. Pennington, K. Yoshida, J. L. Upson, E. Chanclud, C.-H. Wu, T. Langner, A. Maqbool, F. A. Varden,

L. Derevnina, K. Belhaj, K. Fujisaki, H. Saitoh, R. Terauchi, M. J. Banfield, S. Kamoun, Lessons in effector and NLR biology of plant-microbe systems. *Mol. Plant. Microbe Interact.* **31**, 34–45 (2017).

25. J. H. Maidment, M. Shimizu, S. Vera, M. Franceschetti, A. Longya, C. E. Stevenson, J. C. D. la Concepcion, A. Bialas, 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 (2022), p. 2022.06.14.496076, , doi:10.1101/2022.06.14.496076.

26. A. R. Bentham, J. C. De la Concepcion, J. V. Benjumea, S. Jones, M. Mendel, J. Stubbs, C. E. Stevenson, J. H. Maidment, J. Kourelis, R. Zdrzalek, S. Kamoun, M. J. Banfield, Allelic compatibility in plant immune receptors facilitates engineering of new effector recognition specificities. (2022), p. 2022.10.10.511592, , doi:10.1101/2022.10.10.511592.

27. 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*. *Plant Cell*. **21**, 1573–1591 (2009).

28. C. Hamers-Casterman, T. Atarhouch, S. Muyldermans, G. Robinson, C. Hammers, E. B. Songa, N. Bendahman, R. Hammers, Naturally occurring antibodies devoid of light chains. *Nature*. **363**, 446–448 (1993).

29. A. S. Greenberg, D. Avila, M. Hughes, A. Hughes, E. C. McKinney, M. F. Flajnik, A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. *Nature*. **374**, 168–173 (1995).

30. S. Muyldermans, Nanobodies: natural single-domain antibodies. *Annu. Rev. Biochem.* **82**, 775–797 (2013).

31. D. Könning, S. Zielonka, J. Grzeschik, M. Empting, B. Valldorf, S. Krah, C. Schröter, C. Sellmann, B. Hock, H. Kolmar, Camelid and shark single domain antibodies: structural features and therapeutic potential. *Curr. Opin. Struct. Biol.* **45**, 10–16 (2017).

32. J. G. Dingus, J. C. Tang, R. Amamoto, G. K. Wallick, C. L. Cepko, A general approach for stabilizing nanobodies for intracellular expression. *eLife*. **11**, e68253 (2022).

33. A. Kirchhofer, J. Helma, K. Schmidthals, C. Frauer, S. Cui, A. Karcher, M. Pellis, S. Muyldermans, C. S. Casas-Delucchi, M. C. Cardoso, H. Leonhardt, K.-P. Hopfner, U. Rothbauer, Modulation of protein properties in living cells using nanobodies. *Nat. Struct. Mol. Biol.* **17**, 133–138 (2010).

34. P. C. Fridy, Y. Li, S. Keegan, M. K. Thompson, I. Nudelman, J. F. Scheid, M. Oeffinger, M. C. Nussenzweig, D. Fenyö, B. T. Chait, M. P. Rout, A robust pipeline for rapid production of versatile nanobody repertoires. *Nat. Methods*. **11**, 1253–1260 (2014).

35. J. C. De la Concepcion, J. Vega Benjumea, A. Białas, R. Terauchi, S. Kamoun, M. J. Banfield, Functional diversification gave rise to allelic specialization in a rice NLR immune receptor pair. *eLife*. **10**, e71662 (2021).

36. R. Zdrzałek, S. Kamoun, R. Terauchi, H. Saitoh, M. J. Banfield, The rice NLR pair Pikp-1/Pikp-2 initiates cell death through receptor cooperation rather than negative regulation. *PLOS ONE*. **15**, e0238616 (2020).

37. J. Wang, J. Wang, M. Hu, S. Wu, J. Qi, G. Wang, Z. Han, Y. Qi, N. Gao, H.-W. Wang, J.-M. Zhou, J. Chai, Ligand-triggered allosteric ADP release primes a plant NLR complex. *Science*. **364**, eaav5868 (2019).

38. S. P. Dinesh-Kumar, W.-H. Tham, B. J. Baker, Structure–function analysis of the tobacco mosaic virus resistance gene *N*. *Proc. Natl. Acad. Sci.* **97**, 14789–14794 (2000).

39. F. L. W. Takken, W. I. L. Tameling, To nibble at plant resistance proteins. *Science*. **324**, 744–746 (2009).

40. S. Marillonnet, C. Engler, V. Klimyuk, Y. Gleba, Potexvirus-derived replicon (2008), (available at https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2008028661&tab=PCTDESCRIPTION).

41. A. Bendahmane, K. Kanyuka, D. C. Baulcombe, The *Rx* gene from potato controls separate virus resistance and cell death responses. *Plant Cell*. **11**, 781–791 (1999).

42. R. Lu, I. Malcuit, P. Moffett, M. T. Ruiz, J. Peart, A.-J. Wu, J. P. Rathjen, A. Bendahmane, L. Day, D. C. Baulcombe, High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO J.* **22**, 5690–5699 (2003).

43. S. S. Cruz, S. Chapman, A. G. Roberts, I. M. Roberts, D. A. Prior, K. J. Oparka, Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. *Proc. Natl. Acad. Sci.* **93**, 6286–6290 (1996).

44. M. G. Goulden, B. A. Köhm, S. S. Cruz, T. A. Kavanagh, D. C. Baulcombe, A feature of the coat protein of potato virus X affects both induced virus resistance in potato and viral fitness. *Virology*. **197**, 293–302 (1993).

45. M. Luo, L. Xie, S. Chakraborty, A. Wang, O. Matny, M. Jugovich, J. A. Kolmer, T. Richardson, D. Bhatt, M. Hoque, M. Patpour, C. Sørensen, D. Ortiz, P. Dodds, B. Steuernagel, B. B. H. Wulff, N. M. Upadhyaya, R. Mago, S. Periyannan, E. Lagudah, R. Freedman, T. Lynne Reuber, B. J. Steffenson, M. Ayliffe, A five-transgene cassette confers broad-spectrum resistance to a fungal rust pathogen in wheat. *Nat. Biotechnol.* **39**, 561–566 (2021).

46. S. Zhu, Y. Li, J. H. Vossen, R. G. F. Visser, E. Jacobsen, Functional stacking of three resistance genes against *Phytophthora infestans* in potato. *Transgenic Res.* **21**, 89–99 (2012).

47. M. Ghislain, A. A. Byarugaba, E. Magembe, A. Njoroge, C. Rivera, M. L. Román, J. C. Tovar, S. Gamboa, G. A. Forbes, J. F. Kreuze, A. Barekye, A. Kiggundu, Stacking three late blight resistance genes from wild species directly into African highland potato varieties confers complete field resistance to local blight races. *Plant Biotechnol. J.* **17**, 1119–1129 (2019).

48. E. Chae, K. Bomblies, S.-T. Kim, D. Karelina, M. Zaidem, S. Ossowski, C. Martín-Pizarro, R. A. E. Laitinen, B. A. Rowan, H. Tenenboim, S. Lechner, M. Demar, A. Habring-Müller, C. Lanz, G. Rätsch, D. Weigel, Species-wide genetic incompatibility analysis identifies immune genes as hot spots of deleterious epistasis. *Cell*. **159**, 1341–1351 (2014).

49. D. T. N. Tran, E.-H. Chung, A. Habring-Müller, M. Demar, R. Schwab, J. L. Dangl, D. Weigel, E. Chae, Activation of a plant NLR complex through heteromeric association with an autoimmune risk variant of another NLR. *Curr. Biol.* **27**, 1148–1160 (2017).

50. S. Hurni, S. Brunner, D. Stirnweis, G. Herren, D. Peditto, R. A. McIntosh, B. Keller, The powdery mildew resistance gene *Pm8* derived from rye is suppressed by its wheat ortholog *Pm3*. *Plant J.* **79**, 904–913 (2014).

51. Z. Zhang, Y. Wang, Y. Ding, M. Hattori, Structure-based engineering of anti-GFP nanobody tandems as ultra-high-affinity reagents for purification. *Sci. Rep.* **10**, 6239 (2020).

52. Z. Wang, L. Li, R. Hu, P. Zhong, Y. Zhang, S. Cheng, H. Jiang, R. Liu, Y. Ding, Structural insights into the binding of nanobodies LaM2 and LaM4 to the red fluorescent protein mCherry. *Protein Sci.* **30**, 2298–2309 (2021).

53. D. MacLean, besthr - Generating Bootstrap Estimation Distributions of HR Data (2020), (available at https://github.com/TeamMacLean/besthr).

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Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – Original Draft, Writing – Review & Editing, Visualization. Clemence Marchal: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. Andres Posbeyikian: Formal analysis, Investigation, Writing – Review & Editing. Adeline Harant: Investigation, Writing – Review & Editing. Sophien Kamoun: Conceptualization, Writing – Original Draft, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. **Competing interests:** JK, CM, and SK receive funding from industry on NLR biology and have filed a patent on receptor-nanobody fusions (European Patent Application No. 21386064.6). **Data and materials availability**: Pikobody constructs generated for this study will be subjected to MTAs and available upon request. All data are available in the manuscript or the supplementary material .

Supplementary Materials

Materials and Methods

References (54-64)

Figs. S1 to S12

Tables S1 to S6

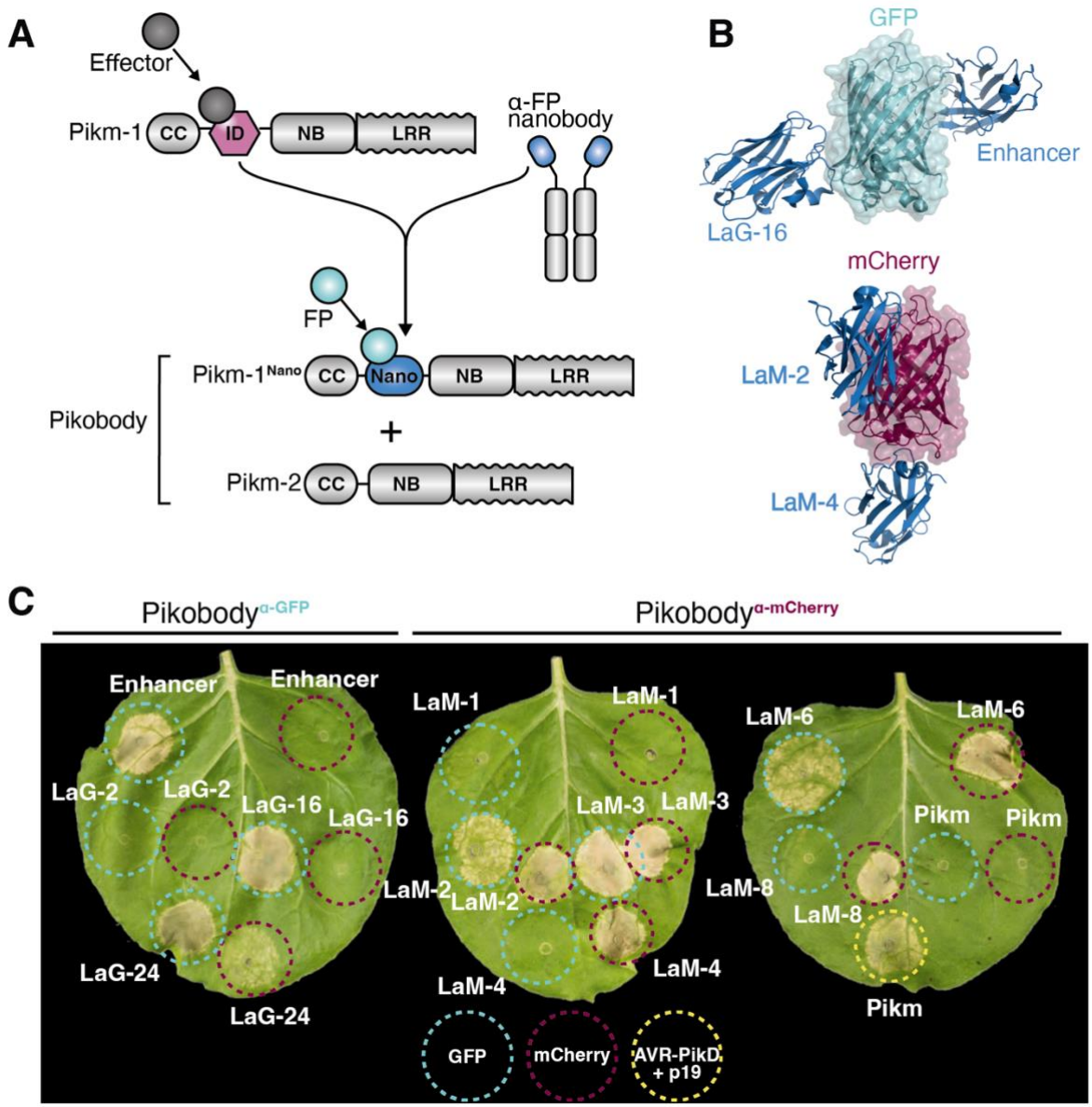

Fig. 1. NLR immune receptor-nanobody fusions trigger a hypersensitive cell death response in presence of the corresponding fluorescent protein antigen. (**A**) Bioengineering of a fluorescent protein (FP)-activated NLR sensor. The integrated HMA domain of the NLR Pikm-1, which is involved in pathogen effector recognition by direct binding, was swapped with nanobodies binding either GFP or mCherry. We coined the term Pikobody for the combination of Pikm-2 with Pikm-1^{Nanobody} fusions. (**B**) Structures of GFP (cyan) or mCherry (magenta) with the GFP-binding nanobodies Enhancer (PDB: 3K1K) (*33*) and LaG-16 (PDB: 6LR7) (*51*), or the mCherry-binding nanobodies LaM-2 (PDB: 6IR2) and LaM-4 (PDB: 6IR1) (*52*), respectively (dark blue). (**C**) Screen for GFP/mCherry recognition by bioengineered Pikobody$^{α\text{-}GFP/α\text{-}mCherry}$ resulting in induction of hypersensitive cell-death response (HR) upon co-expression in *N. benthamiana*. Representative *N. benthamiana* leaves were infiltrated with indicated constructs and photographed 5 days after infiltration (see **Fig. S1** for quantification).

Cyan and magenta dashed circles indicate GFP or mCherry co-infiltration, respectively. The Pikm pair (Pikm-1/Pikm-2) co-infiltrated with AVR-PikD and p19 was used as a positive control for HR (yellow).

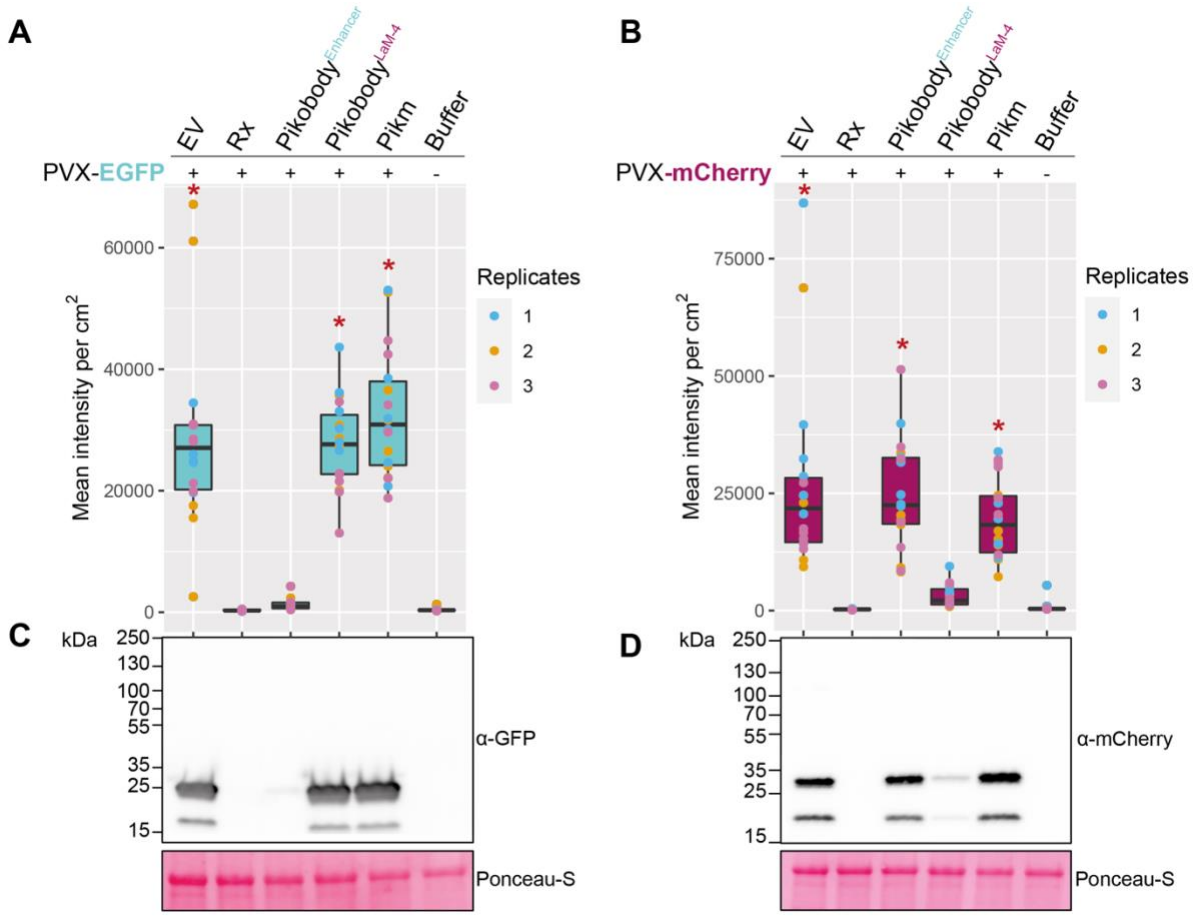

Fig. 2. Pikobodies confer resistance against *Potato virus X* variants expressing matching fluorescent proteins. (**A**-**B**) Specific reduction in fluorescence intensity of PVX expressed EGFP (GFP) or mCherry in the presence of Pikobody^{Enhancer} or Pikobody^{LaM-4}, respectively. GFP (**A**) or mCherry (**B**) mean fluorescence intensity per cm^2 measured in *N. benthamiana* leaves 4 days post-infiltration and used as a proxy for PVX viral load. Boxplots summarize results of three independent replicates with six internal replicates. Asterisks show significant differences between buffer only (no PVX added) and tested constructs in the presence of PVX-GFP (**A**) or PVX-mCherry (**B**) (Dunnett's test, p-value < 0.001). The PVX coat protein recognizing resistance protein Rx was used as a positive control for PVX resistance. (**C**-**D**) Specific reduction of PVX expressed GFP (**C**) or mCherry (**D**) accumulation as proxy to evaluate viral load in the presence of Pikobody^{Enhancer} or Pikobody^{LaM-4}, respectively. Total protein was extracted 4 days after inoculation with PVX variants in the presence of the tested constructs and probed with the appropriate antibodies. Ponceau S staining shows equal protein loading across samples.

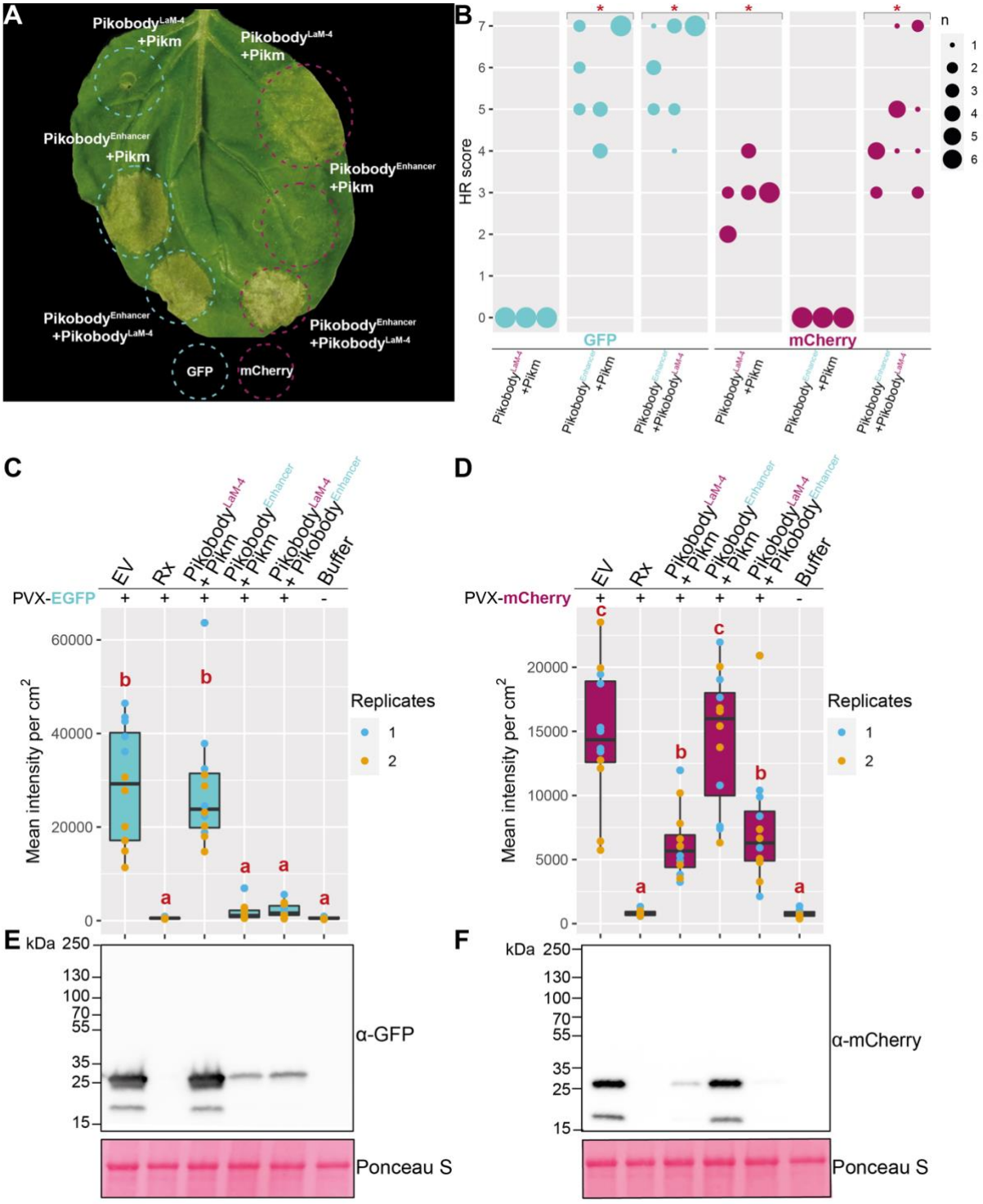

Fig. 3. Stacked Pikobodies result in additive immune recognition and disease resistance. (**A**) Pikobody stacking results in additive immune recognition. Representative *N. benthamiana* leaf infiltrated with indicated constructs. Cyan or magenta dashed lines indicate GFP or mCherry co-expression, respectively. Leaves were photographed 4 days after infiltration. (**B**) HR quantification visualized as dots plot, where the size of a dot is proportional to the number of samples with the same score (n) within the same replicate (1 to 3). The experiment

was repeated three times with six internal replicates. Asterix indicates statistically significant differences as compared to the Pikobody^{Enhancer}+Pikm+mCherry control, as determined by the besthr R package (*53*). (**C-D**) Specific reduction in fluorescence intensity of PVX expressed GFP (**C**) and mCherry (**D**) in the presence of stacked Pikobody^{Enhancer} and Pikobody^{LaM-4}. Mean fluorescence intensity per cm^2 measured in *N. benthamiana* leaves 4 days post-infiltration and used as a proxy for PVX viral load. Boxplots summarize results of three independent replicates with six internal replicates. Letters depict significant differences between treatments as determined by ANOVA followed by Tukey HSD (p-value < 0.05). Specific reduction of PVX expressed GFP (**E**) or mCherry (**F**) accumulation as proxy to evaluate viral load in the presence of stacked Pikobody^{Enhancer} or Pikobody^{LaM-4}. Total protein was extracted 4 days after inoculation with PVX variants in the presence of the tested constructs and probed with the appropriate antibodies. Ponceau S staining shows equal protein loading across samples.

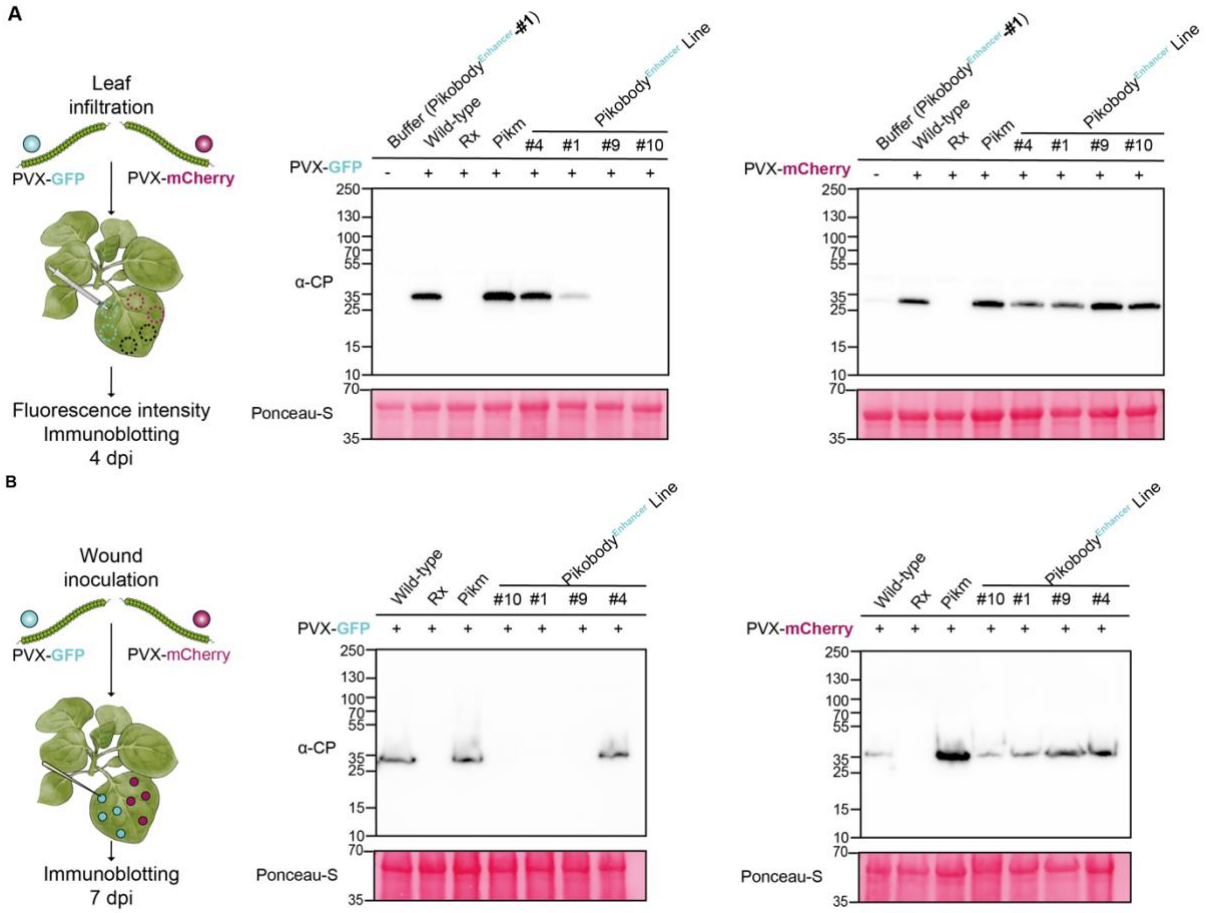

Fig. 4. Transgenic *N. benthamiana* Pikobody^{Enhancer} lines confer specific resistance to PVX-GFP. (**A**) Pikobody^{Enhancer} line #9 displays specific resistance to PVX-GFP delivered by leaf agroinfiltration to an extent similar to Rx, but not to PVX-mCherry. Coat protein accumulation was used to directly measure PVX viral load. For the immunoblot analysis, total protein was extracted 4 days after inoculation of the stated lines with PVX-GFP or PVX-mCherry and probed with the corresponding antibodies. Buffer only (no added PVX) was used as a negative control. Ponceau S staining shows equal protein loading across samples. (**B**) Pikobody^{Enhancer} line #1 and #9 display specific resistance to PVX-GFP delivered by wounding (toothpick inoculation of agrobacterium) to an extent similar to Rx, but not to PVX-mCherry. Immunoblot analysis as in (**A**).