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EDITORIAL

Harnessing nanobodies to expand the recognition spectrum of plant NLRs for diverse pathogens

Ruyi Wang[1] , **Guo-Liang Wang**[2]✉ , **Yuese Ning**[1]✉

[1] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China
[2] Department of Plant Pathology, The Ohio State University, Columbus, OH 43210, USA

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Abstract The strategy to expand the recognition spectrum of plant nucleotide-binding domain leucine-rich repeat (NLR) proteins by modifying their recognition sequences is generally limited and often unsuccessful. Kourelis et al. introduced a groundbreaking approach for generating a customized immune receptor, called Pikobody. This method involves integrating a nanobody domain of a fluorescent protein (FP) into a plant NLR. Their research demonstrates that the resulting Pikobody successfully initiates an immune response against diverse pathogens when exposed to the corresponding FP.

Keywords NLR, Pik, Nanobody, Fluorescent protein, Immunity

The adaptive immune system of animals, which uses structurally similar receptors, including B cell immunoglobulins and T cell receptors, exhibits an almost infinite number of antigen-binding specificities to invading pathogens. By contrast, as plants do not have specialized immune cells or an adaptive immune system, their specific immunity often relies on intracellular nucleotide-binding, leucine-rich repeat (NLR) receptors to recognize the products of cognate avirulence (AVR) effector proteins from pathogens. NLR-mediated immunity signaling requires the formation of paired NLRs, in which a sensor NLR recognizes the pathogen AVR protein and a helper NLR initiates immune signaling.

For example, the rice NLR pairs, RGA5/RGA4 and Pik-1/Pik-2, confer resistance to isolates of the fungal pathogen *Magnaporthe oryzae* carrying the cognate AVR genes (Zhai et al. 2011; Cesari et al. 2013). Notably, the heavy-metal-associated (HMA) domain in RGA5 and Pik-1 directly interacts with the cognate effectors for recognition (Cesari et al. 2013) (Fig. 1, left image). Structure-guided mutagenesis of the RGA5 HMA domain led to the recognition of the non-cognate effector AVR-Pib and defense activation (Liu et al. 2021). The *Pik* gene pair has multiple alleles, which display specific resistance to *M. oryzae* isolates with different cognate *AVR-Piks*. Similarly, mutagenesis of two adjacent amino acids in the Pikp HMA domain expanded its recognition profile to *M. oryzae* isolates containing AVR-Pik variants (De la Concepcion et al. 2019). These findings suggest that the HMA domain is, indeed, responsible for the recognition of different AVR–NLR pairs. However, the modified Piks are still unable to recognize every AVR-Pik variant. Thus, modification of the plant NLR repertoire, for pathogen recognition, has limited potential for expanding the recognition of AVRs of a wider range of pathogens.

Compared with the conventional immunoglobulin G (IgG) antibodies present in most mammals, the Camelidae (with extant members such as the camels) possess unique IgG antibodies that lack the complete light-chain polypeptide and only contain the N-terminal variable domain of the heavy chain, known as VHH. These VHH domains, which specifically recognize their target antigens, are commonly referred to as nanobodies (Muyldermans 2013). Nanobodies have a molecular weight of 12–15 kDa, which is less than 10% that of conventional

✉ Correspondence: wang.620@osu.edu (G.-L. Wang), ningyuese@caas.cn (Y. Ning)

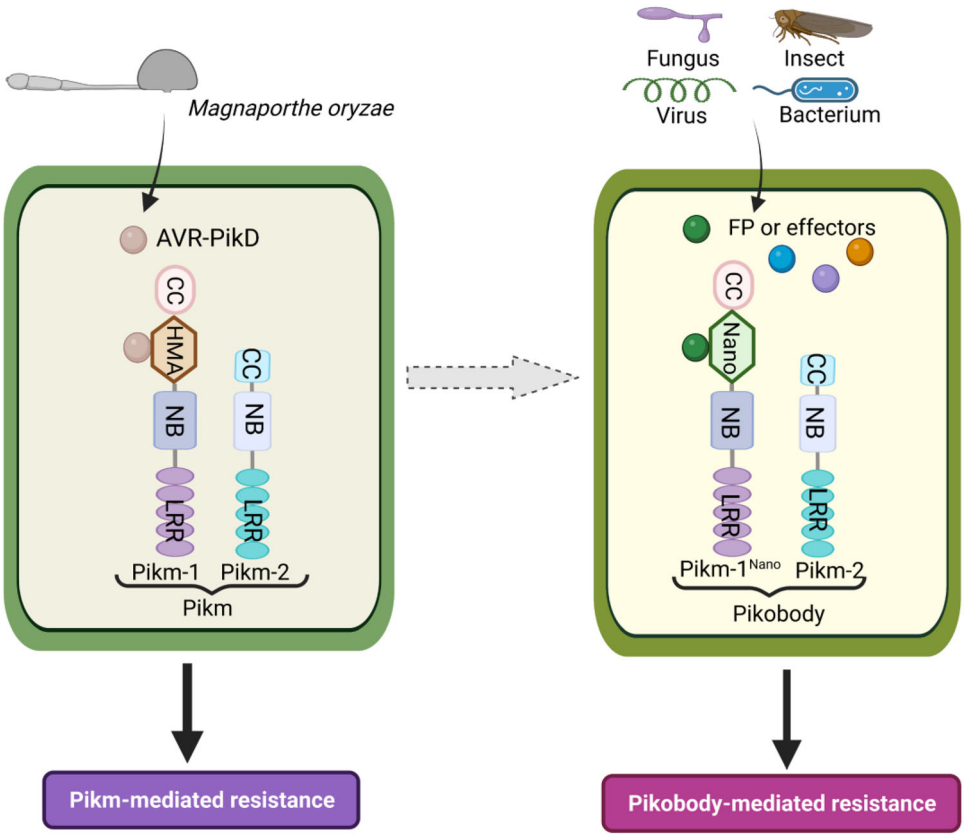

Fig. 1 Expression of Pikm-1–nanobody and Pikm-2 leads to broad-spectrum resistance against pathogens containing the corresponding fluorescent proteins or effectors. In rice, a paired NLR protein including Pikm-1 and Pikm-2 displays specific resistance against *M. oryzae* isolates containing the cognate AVR-PikD (left image). Expression of the Pikobody confers plant resistance to pathogens or insects containing the corresponding fluorescent proteins (FPs) or effectors (right image). The integrated HMA domain of Pikm-1 is swapped with the nanobody-binding FPs or cognate effectors from a fungus, bacterium, virus, or insect. The resulting transgenic plants show resistance to pathogens or insects harboring the corresponding fluorescent protein sequence in their genomes or pathogens or insects containing the corresponding effectors. CC, coiled-coil domain; NB, nucleotide-binding domain; LRR, leucine-rich repeat; Nano, nanobody

antibodies. Despite their smaller size, nanobodies exhibit similar antigen-binding characteristics and may have a more compact structure, allowing better access to hidden antigens (Muyldermans 2013). Fluorescent proteins (FPs), including green fluorescent protein (GFP) and mCherry, are widely used in molecular biological research. The high affinity and small sizes of the GFP and mCherry binding nanobodies from the Camelidae are ideal tools for detecting target FP-fusion proteins in live cellular compartments (Kirchhofer et al. 2010).

Taking advantage of the role of the HMA domain in AVR-Pik/Pik recognition and the antigen accessibility of the nanobody, Kourelis et al. at the Sainsbury Laboratory developed a Pikm-1-nanobody fusion protein in which the Pik HMA domain was exchanged with GFP and mCherry FP nanobodies (Kourelis et al. 2023). Transient co-expression of Pikm-2, Pikm-1–nanobody fusions, and their matching FP in *Nicotiana benthamiana* leaves produced a hypersensitive response (HR), which resembled that generated using a combination of Pikm-1, Pikm-2, and AVR-PikD, indicating that the transiently expressed Pikm-1–nanobody fusions can recognize FPs and activate Pikm-2-dependent immunity (Fig. 1, right image).

It is worth noting that a P-loop dead mutation in Pikm-2, with the Pikm-1–nanobody and the corresponding FP, failed to produce a HR. These results suggest that the P-loop in Pikm-2 is required for Pikobody activity and, further, that the combination of the engineered Pikm-1–nanobody fusions and Pikm-2 function via a similar mechanism to that of other NLRs. Remarkably, the Pikobody was activated after recognizing FPs without any pathogen effectors, suggesting that the FPs can act as synthetic AVRs when they co-localize with the Pikobody within plant cells.

An engineered PBS1 protein, whose cleavage site was replaced by cleavage sites recognized by different *Pseudomonas syringae* protease-type AVRs, enabled the singleton NLR protein, RPS5, to trigger immunity to

another *Pseudomonas* pathovar (Kim et al. 2016). Remarkably, in addition to bacterial pathogen-secreted protease-type AVRs, the engineered PBS1 protein could also be cleaved by protease-type AVRs from *Turnip mosaic virus* (Kim et al. 2016), demonstrating that RPS5 activation-mediated immunity can be switched from bacterial to viral immunity. Similarly, Kourelis et al. tested whether Pikobodies can effectively activate immunity after infection by *potato virus X* (PVX), which causes a mild mosaic disease in potato, *N. benthamiana* and other solanaceous plants. The 6.4-kb *PVX* genome contains five open reading frames, one of which encodes the coat protein that can be recognized by the NLR resistance protein Rx. When a FP sequence in the virus genome was co-expressed with a Pikobody in *N. benthamiana* leaves, the Pikobody was successfully activated and produced *Rx*-like resistance against *PVX-FP* (Kourelis et al. 2023) (Fig. 1, right image). Notably, the Pikobody could even confer resistance against *PVX-FP* variants harboring mutations in the *PVX* coat protein that allow them to evade *Rx*-mediated immunity (Kourelis et al. 2023). The authors generated stable Pikobody transgenic lines in *N. benthamiana* and demonstrated that the Pikobody conferred resistance to *PVX*.

These results suggest that Pikobodies originating from rice can confer resistance in other plants and that Pikobody-mediated immunity can switch from fungal to viral immunity. It will be interesting to determine whether rice plants harboring the Pikobody can be activated by rice viruses expressing GFP or mCherry and whether virus-activated rice Pikobody-mediated immunity confers broad-spectrum disease resistance against fungal and bacterial pathogens.

To ascertain whether stacking of GFP and mCherry Pikobodies would increase resistance to pathogens, Kourelis et al. co-expressed Pikobody$^{\alpha-\mathrm{GFP}}$ and Pikobody$^{\alpha-\mathrm{mCherry}}$ in *N. benthamiana*. The infiltrated plants still retained independent recognition specificities for their corresponding FPs and produced a HR in the presence of both FPs, indicating that Pikobody stacking leads to additive recognition capacities and, possibly, enhanced resistance compared to the use of individual Pikobodies. As Pia and Pik have similar HMA domains for AVR-binding, but show different resistance spectra, replacing the RGA5 HMA domain with a GFP or mCherry nanobody in rice plants can generate "Piaobody" rice plants. Perhaps the stacking of Pikobodies and Piaobodies may further expand broad-spectrum resistance against *M. oryzae* in rice.

Although this proof of concept was successful in the heterologous model plant, *N. benthamiana*, it remains unknown whether the Pikobody will provide resistance in rice against *M. oryzae*. To explore this possibility, one approach is to introduce the Pikobody plasmids into a non-*Pikm*-containing rice cultivar and then infect the resulting transgenic plants with an *M. oryzae* isolate expressing either the *GFP* or *mCherry* gene without the *AVR-PikD* effector. Another method involves replacing the HMA domain in the rice *Pikm* plants with the nanobody sequence of *GFP* or *mCherry* (Lu et al. 2020) and subsequently inoculating the modified plants with an FP-containing *M. oryzae* isolate. Additionally, as nanobodies can be generated to bind virtually any antigen, a promising approach could involve using core effectors from *M. oryzae* to raise nanobodies in the Camelidae and substitute the corresponding nanobody sequences for the HMA domain of Pikm-1 (Fig. 2). Upon infection with *M. oryzae* carrying the core effector, the genetically modified rice plants expressing the respective Pikobody should exhibit immune responses during the infection process.

Some limitations must be overcome before Pikobody-based technology can be used for plant disease control. First, as a direct interaction between the Pikobody and the corresponding FP during the early stages of infection in the cytosol is critical for defense activation, how enough FPs can be delivered to the intracellular space remains to be determined. Perhaps nanoparticle-mediated delivery systems could be used for FP delivery, such as chitosan-complexed single-walled carbon nanotubes designed to deliver plasmid DNA into plant cells (Kwak et al. 2019); this concept remains to be explored.

An alternative method might be to express the FP gene under the control of a pathogen-inducible promoter in transgenic plants. As mentioned above, some ubiquitous pathogen effectors could be selected to raise nanobodies in Camelids, and the corresponding nanobody sequences could then be inserted in the Pikobody. However, whether the amount of the effector protein, secreted from the pathogen into plant cells, is sufficient to trigger strong defense responses to inhibit pathogen invasion requires detailed experimentation. In a second approach, the NLR-based Pikobody strategy could, potentially, be used in both biotrophic and semi-biotrophic pathosystems. Whether it would also function in necrotrophic pathosystems remains to be confirmed, as most host resistance responses against necrotrophs are mediated by non-NLR proteins.

Here, it should be mentioned that, as NLR over-activation always results in a trade-off between growth and resistance, the effect of Pikobody-mediated immunity on crop yields should be carefully examined in the field. Furthermore, non-specific binding to the nanobody domain by other unrelated proteins should be tested, under different abiotic and biotic stress conditions,

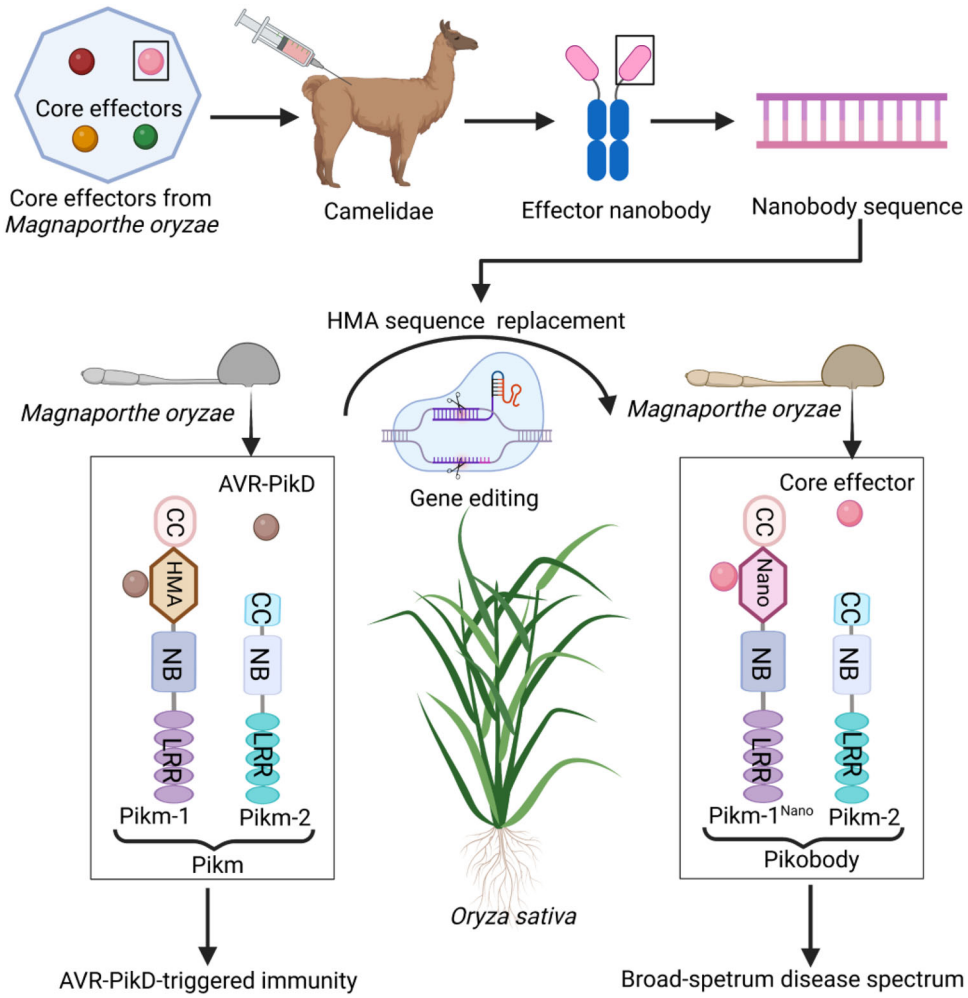

Fig. 2 Harnessing nanobodies to develop broad-spectrum disease resistant rice. Using core effectors from *M. oryzae* to raise nanobodies in the Camelidae and substitute the corresponding nanobody sequences for the HMA domain of Pikm-1 using gene editing approach. Once *M. oryzae* carrying the core effector infects rice, the genetically modified rice plants, expressing the corresponding Pikobody, should exhibit immune responses during infection

prior to the use of this strategy in crop production. Nevertheless, Kourelis et al. pioneered the use of nanobodies and the incorporation of new integrated domains into NLRs to generate broad-spectrum resistance in plants. This approach, combined with recent advances in engineering helper NLRs to evade immune suppression to resurrect disease resistance (Contreras et al. 2023), has the potential to develop synthetic receptors against many pathogens for deployment in different crops.

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Data availability Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors have no conflict of interests to declare. Author Guo-Liang Wang was not involved in the journal's review of the manuscript.

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