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LETTER TO THE EDITOR

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Engineering nucleotide-binding leucine-rich repeat immune receptors to achieve broad-spectrum disease resistance in plants

Plant diseases cause up to 30% of global crop losses annually, posing a severe threat to food security. To meet the growing food demand, developing disease-resistant crops is urgent for modern agriculture. Most plant disease resistance (*R*) genes encode nucleotide-binding leucine-rich repeat (NLR) proteins, which are intracellular immune receptors composed of variable N-terminal domains, a central conserved NB-ARC domain (nucleotide-binding adaptor shared with APAF-1, R-proteins and CED-4), and a C-terminal leucine-rich repeat (LRR) domain. Three major groups of plant NLRs are defined according to their diverse N-termini: coiled-coil (CC) domain-containing NLRs (CNLs), Toll-like/interleukin-1 receptor (TIR) domain-containing NLRs (TNLs), and RESISTANCE TO POWDERY MILDEW 8-like CC (CC_R) domain-containing NLRs (RNLs). Generally, CNLs and TNLs act as sensor NLRs to detect pathogen effectors, whereas RNLs, also termed helper NLRs, function downstream of many sensor NLRs to transmit immune signals.[1] Sensor NLRs recognize specific pathogen effectors either directly or indirectly and then trigger effector-triggered immunity (ETI), a robust immune response which often manifests as the hypersensitive response (HR). Certain NLRs mediating antiviral immunity even confer extreme resistance without visible HR. Given its essential role in plant immunity, NLR transferring mediated by conventional breeding or transgenesis has been deployed for disease resistance improvement in susceptible cultivars. However, this strategy is often limited by resistance resources, immune background of the recipients, potential growth-defense trade-offs, and the lack of durability.[2] Advances in understanding NLR structure and activation mechanisms now enable the design of NLRs with new or expanded recognition profiles.[2–4] Here, we will summarize these pioneering studies of NLR engineering for broad-spectrum and durable disease resistance in plants.

1 | ENGINEERING OF LRR DOMAIN

The LRR domain of sensor NLRs is demonstrated to play a crucial role in direct recognition of pathogen effectors, making it a promising target for engineering NLRs with altered recognition profiles (Figure 1).[5–8] Random mutagenesis of the Rx LRR identified Rx variants conferring resistance not only against diverse potato virus X strains but also against the distantly related poplar mosaic virus.[5] Similarly, random mutagenesis of the Tm-2^2 LRR domain yielded a variant (S723Y/N744D) with enhanced effector binding affinity and extended resistance against the emerging tomato brown rugose fruit virus.[6] Random mutagenesis targeting both the LRR and the N-terminal *Solanaceae*-specific domain (SD) of Sw-5b also successfully broadened its resistance spectrum against tomato spotted wilt virus isolates.[7] Structural insights into NLR resistosomes now enable precise modifications of effector-binding interfaces. For instance, based on the structure of the wheat Sr35 resistosome, LRR domain swapping or targeted mutagenesis of the closely related Sr35 homologs, TaSH1 from wheat and HvSH1 from barley, has generated new variants of those orphan NLRs that can recognize AvrSr35.[8]

2 | ENGINEERING OF DECOYS

NLRs can recognize pathogen effectors indirectly via host decoy proteins that mimic effector targets. Engineering these decoys holds potential to alter NLR recognition specificity. A key example is the modification of the *Arabidopsis* decoy PBS1 (Figure 1). Normally, the *Arabidopsis* NLR resistance protein RPS5 is activated when the decoy PBS1 is cleaved by *Pseudomonas syringae* protease effector AvrPphB. Substituting the protease cleavage site (PCS) of PBS1 with PCSs recognized by other pathogen proteases, such as

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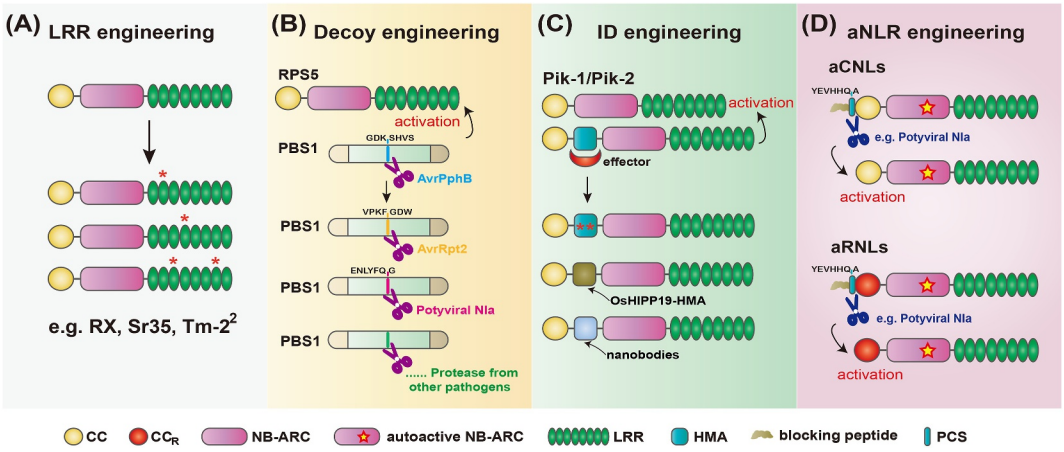

FIGURE 1 Strategies of NLR engineering for broad-spectrum and durable disease resistance. (A) LRR engineering by random mutagenesis or structure-guided mutagenesis targeting effector-binding regions in LRR domains. Asterisks indicate mutations. (B) Decoy engineering. RPS5 is activated when the decoy protein PBS1 is cleaved by the effector protease, AvrPphB. By swapping the PCS in PBS1 with proteolytic sites that can be recognized by other pathogen-secreted proteases, RPS5 can be activated to trigger the immune response against these pathogens. (C) ID engineering. HMA in the Pik-1 of the paired Pik NLRs is an ID responsible for detecting effector proteins secreted by the fungal pathogen *Magnaporthe oryzae*. The recognition specificity of Pik-1 can be expanded by structure-guided mutagenesis of HMA, domain swapping by HMAs of other host targets of effectors, or by nanobodies that can recognize pathogen proteins. Asterisks indicate mutations. (D) aNLR engineering. Under normal conditions, the autoactivity of aCNLs or aRNLs is suppressed by the N-terminal blocking peptide coupled with PCS. Upon invasion, protease-secreted by pathogens will cleave the chimeric aNLRs and release aNLRs to trigger strong innate immunity in hosts. aNLRs, autoactive NLRs; CC, coiled-coil; CNLs, CC domain-containing NLRs; HMA, heavy metal-associated domain; ID, integrated domain; LRR, leucine-rich repeat; NB-ARC, nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4; NLR, nucleotide-binding leucine-rich repeat; PCS, protease cleavage site; and RNLs, CC_R domain-containing NLRs.

bacterial AvrRpt2 or potyviral NIa protease, enabled RPS5 activation, upon infection by pathogens expressing those effectors, to confer resistance.[9] Whereas theoretically adaptable to any pathogen utilizing a protease, this approach is constrained to hosts possessing similar PBS1-guarding NLR proteins.

3 | ENGINEERING OF IDs IN NLRs

Some NLRs have integrated decoy domains (IDs) that mimic effector targets and mediate direct effector recognition.[10] Engineering of IDs for expanded recognition is well-studied in the rice NLR pairs Pik-1/Pik-2, which cooperatively confer resistance to *Magnaporthe oryzae* strains (Figure 1). Effector binding to the ID, a heavy metal-associated (HMA) domain within sensor Pik-1 alleles, is required to activate Pik-mediated immunity. Two parallel approaches, structure-guided mutagenesis of Pikp-1 HMA domain or replacing it with modified OsHIPP19-HMA from another host target of Avr-Pik, were demonstrated to be practicable for generating transgenic rice with expanded resistance to *M. oryzae* expressing Avr-PikC or Avr-PikF.[11] Furthermore, swapping the Pik-1 HMA domain with nanobodies that bind fluorescent proteins generated pikobodies conferring resistance against FP-tagged viruses in plants.[12] This "pikobodies" strategy holds the potential for tailoring crop disease resistance against any pathogen or pest that delivers effectors inside plant cells, provided that deleterious autoimmunity is avoided.[13]

4 | ENGINEERING OF aNLRs

Beyond modifying direct effector-binding NLR domains or decoys, exploiting effector-mediated derepression of autoactive NLRs (aNLRs) offers another strategy for altering recognition. The *Arabidopsis* TIR-NLR pair RRS1-R/RPS4 recognizes bacterial effectors AvrRps4 from *P. syringae* and PopP2 from *Ralstonia solanacearum*. RRS1-R represses RPS4 autoactivity, whereas its RRS1-R^{slh1} mutant causes RPS4-dependent autoimmunity. A system composed of an autoactive RRS1-R^{slh1}, an RPS4, and an inactive RRS1 with effector-dependent degron was designed in *Arabidopsis*, where autoimmunity of RRS1-R^{slh1} is believed to be precisely derepressed upon effector recognition. However, transgenic *Arabidopsis* expressing an RRS1-R with a SAP05-dependent degron showed only delayed symptoms, not full resistance, after phytoplasma infection.[14] A recent breakthrough presents a simpler and more powerful aNLR engineering strategy.[3] Activated CNLs or CC_R-NLRs (RNLs) form resistosomes, which act as plasma membrane calcium channels to initiate ETI.[1] A short N-terminal helix in the CC/CC_R domain of CNLs/RNLs is crucial for their pore-forming activity, and N-terminal fusions preceding the CC/CC_R domain may impede this

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function.[3] Wang et al. designed chimeric aNLRs inactivated by an N-terminal blocking polypeptide coupled with a conserved pathogen-derived PCS.[3] Upon infection, pathogen proteases cleave the chimeric aNLRs, releasing the functional aNLR to trigger a potent immune response (Figure 1). The chimeric autoactive CNL Tm-2^2 coupled with a 7-residue conserved NIa PCS of potato virus Y (PVY), HA-PCS^{PVY}-aTm-2^2, or the chimeric autoactive RNL AtNRG1.1 coupled with the PVY NIa PCS, HA–PCS^{PVY}–aAtNRG1.1, conferred broad, complete, or even extreme resistance against multiple potyviruses in transgenic tobacco plants. Using tandem PCSs from different viruses, such as the tobacco etch virus and PVY, the resistance spectrum engineered by aNLRs can be further expanded. Moreover, transgenic soybeans expressing chimeric aAtNRG1.1 with a PCS from soybean mosaic virus (SMV), one of the most devastating pathogens of soybeans worldwide, conferred complete resistance to SMV.

5 | PROSPECTIVE

Current strategies for NLR engineering largely focus on effector-binding interfaces that are heavily reliant on the structural knowledge of NLR-effector complexes. Applications of PBS1 engineering or "pikobodies" are limited by the recipient's immune background or the availability of appropriate nanobodies that recognize effectors. In contrast, the latest aNLR engineering provides a brand-new, simple, and potent approach for creating crops with broad-spectrum disease resistance against variable pathogens. It requires only a single chimeric construct composed of an autoactive CNL or RNL and an N-terminal blocking peptide coupled with a pathogen-derived PCS. Given that many pathogens/insects can secrete proteases to aid their infection/infestation, resistance to any pathogens/insects could be tailored by swapping PCSs in the chimeric aNLRs. This resistance is likely durable, as protease mutations preventing cleavage would likely compromise pathogen/pest fitness. The robust resistance of aCNLs/aRNLs stems from their conserved Ca^{2+}-channel activity, suggesting the wide applicability of this strategy across crops. Coupled with CRISPR-Cas genome editing, aNLR engineering could potentially condition the autoactivity of endogenous NLRs to enhance resistance. In summary, aNLR engineering open new avenues for breeding crops with broad-spectrum and durable disease resistance.

AUTHOR CONTRIBUTIONS

Yan Wang: Writing—original draft; writing—review and editing; conceptualization; funding acquisition; resources. **Yuzhen Mei**: Writing—original draft; writing—review and editing. **Fangfang Li**: Conceptualization; writing—original draft; funding acquisition; writing—review and editing; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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DATA AVAILABILITY STATEMENT

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

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