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Current Opinion in Plant Biology

Emerging principles in the design of bioengineered made-to-order plant immune receptors

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Abstract

Crop yield and global food security are under constant threat from plant pathogens with the potential to cause epidemics. Traditional breeding for disease resistance can be too slow to counteract these emerging threats, resulting in the need to retool the plant immune system using bioengineered made-to-order immune receptors. Efforts to engineer immune receptors have focused primarily on nucleotide-binding domain and leucine-rich repeat (NLR) immune receptors and proof-of-principles studies. Based upon a near-exhaustive literature search of previously engineered plant immune systems we distil five emerging principles in the design of bioengineered made-to-order plant NLRs and describe approaches based on other components. These emerging principles are anticipated to assist the functional understanding of plant immune receptors, as well as bioengineering novel disease resistance specificities.

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Current Opinion in Plant Biology xxxx, **xxx**:xxx

This review comes from a themed issue on **Plant Biotechnology**

Edited by **Nicola J. Patron** and **Steven J. Burgess**

For a complete overview see the Issue and the Editorial

Available online xxx

https://doi.org/10.1016/j.pbi.2022.102311

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Keywords

NLR, NBS-LRR, NB-LRR, NLR-ID, NLR engineering, Synthetic biology.

Introduction

The plant immune system is effective in preventing serious disease caused by most pathogens. When certain pathogens, however, manage to overcome the plant immune system they can present a huge threat towards agriculture. In a changing world with a fragile food infrastructure this can result in shortages and even famine and armed conflicts [1]. Natural disease resistance (*R*) genes have traditionally been bred into commercial varieties of crops to provide effective immunity against novel pathogens. Most of these *R* genes are either cell-surface receptors, or intracellular nucleotide-binding domain and leucine-rich repeat −containing proteins (NLRs) [2].

Given that introducing novel *R* genes into commercial varieties of crops is time-consuming and can be overcome once deployed, there is a desire for a more precise engineering of the plant immune system. Current efforts to retool the plant immune system have predominantly focused on NLRs. NLRs are receptors involved in immunity across all kingdoms of life [3–5]. Plant NLRs are characterized by a central NB-ARC (nucleotide-binding domain shared with APAF-1 [apoptotic peptidase activating factor 1], various R proteins, and CED-4 [cell death-4]) domain, and are generally associated with a C-terminal leucine-rich repeat (LRR) domain [6]. Typically, it is thought that effector binding to the C-terminal LRR domain reduces intramolecular autoinhibition by the LRR domain [7]. This allows the NB-ARC domain to mediate the intramolecular activation of the NLR protein by exchanging adenosine diphosphate (ADP) for adenosine triphosphate (ATP) in the nucleotide-binding (NB) pocket [8,9]. This results in the oligomerization of NLR proteins into structures known as resistosomes and activation of the variable N-terminal domains of the NLRs [9–12]. In seed plants, the variable N-terminal domains are either Toll/Interleukin-1 Receptor (TIR) domains, which are enzymatically activated upon resistosome formation [10,11,13,14], or various types of coiled-coil (CC) domains, which act as cation channels upon resistosome formation [12,15,16]. Ultimately, activation of the N-terminal domains results in an effective immune response which is often accompanied by a specific type of programmed cell-death known as the hypersensitive cell-death response.

Currently, bioengineered plant immune receptors have been generated by targeted mutagenesis guided by structural, evolutionary, or mechanistic information, random mutagenesis, or domain shuffling. Based upon a near-exhaustive literature search of these bioengineered NLR immune receptors (**S1 Table**), we distilled five

emerging principles in the design and engineering of made-to-order plant NLRs (Figure 1).

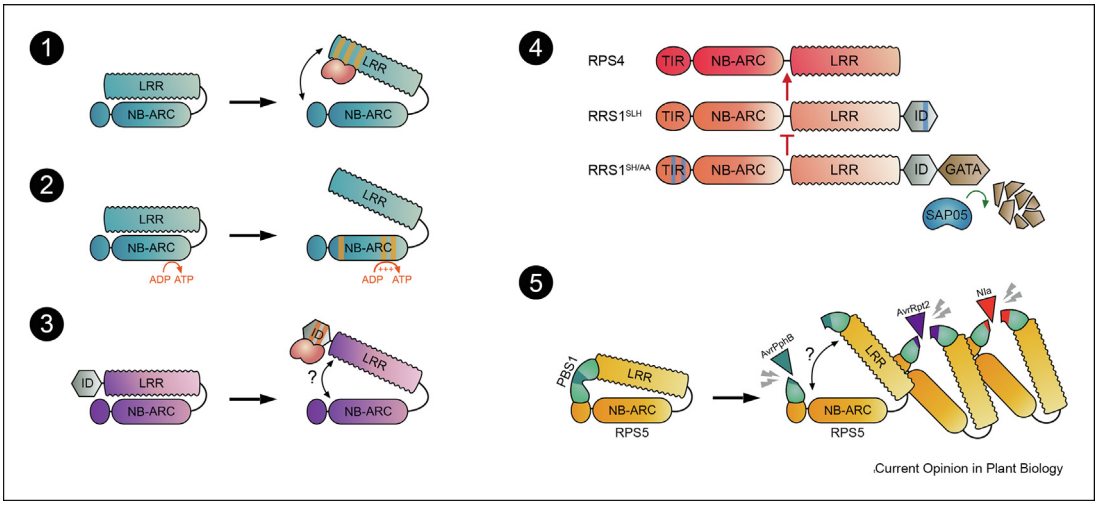
Principle 1: engineering ligand-binding induced steric clashes releases NLR autoinhibition

Structural studies on the activation mechanisms of plant NLRs provide crucial information on how to generate bioengineered NLRs. The wheat NLR Sr35, for example, provides immunity to AvrSr35 expressing strains of the fungal pathogen *Puccinia graminis* f. sp. *tritici* that causes wheat stem rust disease [17,18]. Comparison of the structure of the activated Sr35 resistosome to a structural model of inactive Sr35 reveals two common principles of NLR activation: 1) the N-terminal part of the Sr35 LRR is involved in stabilizing both the inactive and activated Sr35 structure, and 2) direct binding of AvrSr35 to the C-terminal ascending lateral side of the Sr35 LRR domain would cause a "steric clash" between the Sr35 LRR and NB domain. The steric clash is hypothesized to result in dislodging of the NB domain, allowing for a switch of ADP to ATP in the NB-ARC domain resulting in assembly of the Sr35 resistosome [12]. Both features appear to be conserved across plant NLRs [12].

The steric clash model implies that, in principle, all that is required to engineer a plant NLR to respond to a novel effector is to engineer a binding site at the ascending lateral side of the LRR domain. Indeed, mutations in five different NLRs—*Ta*SH1, *Hv*SH1, Sr33, Rx, and R3a—resulting in altered or enhanced recognition spectra appear to follow this model (Figure 2). Two homologs of Sr35 previously unable to respond to AvrSr35, *Ta*SH1 and *Hv*SH1, were engineered to respond to AvrSr35 by targeted mutagenesis of residues within the LRR based on structural and evolutionary information [12] (Figure 2a). Similarly, Sr33, a homolog of the Sr50 NLR which recognizes AvrSr50, was engineered to respond to AvrSr50 following an evolution-guided approach which revealed an evolutionary "footprint" of effector-binding [19] (Figure 2b). Given that the cognate effectors of *Ta*SH1, *Hv*SH1, and Sr33 are unknown, it cannot be determined whether the engineered versions of these NLRs have lost the capacity to respond to their cognate ligands.

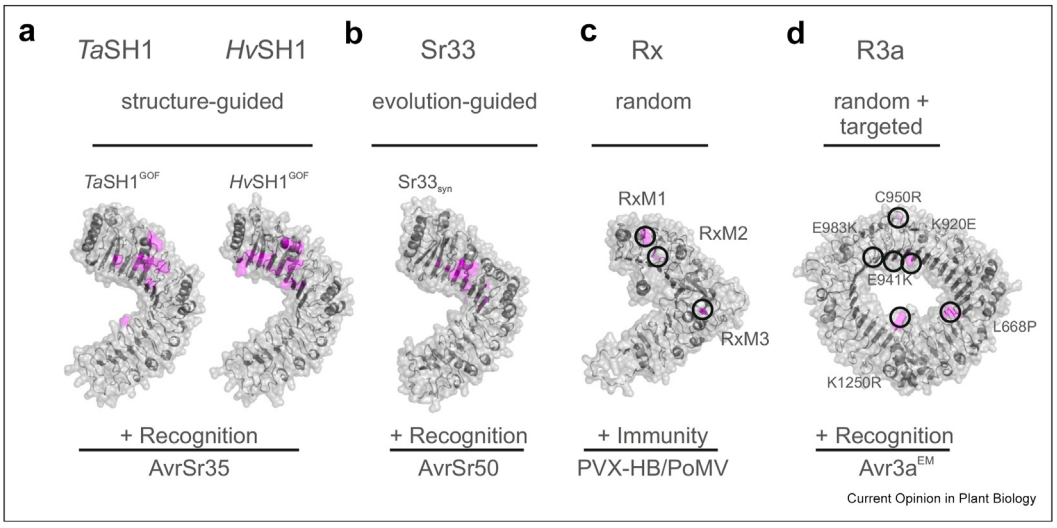
No prior information is necessarily required to engineer NLRs, as the recognition spectrum of both Rx [20] and R3a [21] has been extended by random mutagenesis. In the case of Rx, three independent single amino acid mutations in the LRR confer immunity towards a resistance-breaking strain of *potato virus X* (PVX) [20] (Figure 2c). How exactly these mutations expand the Rx recognition spectrum remains to be determined, as no direct interaction has been found between Rx and the PVX coat protein, and Rx itself does not appear to oligomerize in the presence of its coat protein ligand [22,23]. Finally, in the case of R3a, six independent amino acid substitutions in the LRR were identified by random mutagenesis and could also confer recognition of an immune-evading allele of the potato blight pathogen *Phytophthora infestans* effector Avr3a [21] (Figure 2d). The steric clash model indicates that these amino acid

Figure 1



Five emerging principles in the design and engineering of bioengineered made-to-order plant NLR immune receptors. Based upon a near-exhaustive literature search we define five emerging principles in the design and engineering of made-to-order plant immune receptors: 1) engineering ligand-binding induced steric clashes between the LRR and NB-ARC domains alters and expands immune recognition, 2) sensitizing mutations resulting in reduced autoinhibition throughout the NLR expand immune recognition, 3) mutations and swaps of integrated domains alters and expands immune recognition, 4) engineered gene networks in which autoactivity is conditionally derepressed can generate novel immune recognition, 5) decoy engineering to alter immune recognition. Therefore, bioengineering NLRs can involve new or expanded pathogen recognition specificities (1, 3, 4, 5), or sensitized NLRs (2).

Figure 2



Engineering effector-recognition sites at the ascending lateral side of the LRR. Structural models of the LRR region of the NLRs **a**) *Ta*SH1 and *Hv*SH1, **b**) Sr33, **c**) Rx, and **d**) R3a, generated using AlphaFold2 [24] as implemented in ColabFold [25]. Amino acid substitutions are highlighted in magenta and accumulate at the ascending lateral side of the LRR. **a**) Structure-guided engineering of *Ta*SH1^{GOF} (*Ta*SH1$^{D609G/Y728F/I755K/Q756R/L804W/Q810E/R857W/D731R}$) and *Hv*SH1^{GOF} (*Hv*SH1$^{Y727F/Q752P/G754K/Q755R/Q801E/R809E/W835I/R856W/R917D/P919W}$) to confer recognition of AvrSr35 [12]. **b**) Evolution-guided engineering of an AvrSr50-recognizing Sr33$_{syn}$, which contains 12/26 variant residues (Sr33$^{Q707K/Q707K/I735S/C738Y/E767W/P795W/T796P/L818W/E820K/V840M/Y843E/C866V}$) found within the region between Sr33 and Sr50 found to be highly variable [19]. **c**) Error-prone PCR of the Rx LRR region was used to generate RxM1 (Rx^{N846D}), RxM2 (Rx^{N796D}), and RxM3 (Rx^{L607P}) conferring recognition of the coat protein of, and immunity to, the resistance-breaking *potato virus X* (PVX)-HB strain as well as the distantly related *poplar mosaic virus* (PoMV) [20]. RxM1 displays a trailing necrosis phenotype with PoMV, indicative of delayed immune activation [20]. **d**) Random mutagenesis of the entire coding sequence of R3a to identify mutants with an enhanced recognition spectrum also conferring recognition to the immune-evading Avr3a^{EM} allele of Avr3a. R3a containing either L668P, K920E, E941K, C950R, E983K, or K1250R in the LRR can recognize Avr3a^{EM} in addition to Avr3a^{KI} [21]. R3a^{L668P} can also recognize a *Phytophthora capsici* homolog of Avr3a (*Pc*AVR3a4) [21].

substitutions in Rx and R3a may be directly involved in effector recognition resulting in a ligand-induced steric clash with the NB domain, although it might be that these mutations expand the recognition spectrum via another mechanism.

Principle 2: sensitizing mutations throughout the NLR can expand recognition

Given that NLRs are activated by release of auto-inhibition, mutations resulting in reduced auto-inhibition yield sensitized NLRs with potentially expanded recognition spectra. Indeed, such sensitizing mutations, sometimes combined with other mutations, enhance the immune spectrum of at least four different NLRs: Rx, Sw-5b, R3a, and I-2.

For Rx, mutagenesis of the LRR resulted in one mutant containing a single amino acid substitution in the N-terminal part of the LRR likely involved in stabilizing active and inactive conformations of Rx [12,20]. This Rx mutant appears to be more "trigger-happy", which explains its expanded immune recognition [20]. While two of the Rx mutants described above confer effective immunity against the resistance-breaking strain of PVX and the highly divergent *poplar mosaic virus* (PoMV), the third Rx mutant displays a trailing necrosis phenotype indicative of delayed immune activation in response to PoMV [20]. To sensitize this Rx mutant, one to two amino acid substitutions identified by random mutagenesis were introduced in the NB-ARC domain [26]. This stepwise mutated Rx could provide full immunity towards PoMV [26]. The mutations in the NB-ARC domain likely enhance affinity for ATP, or conversely reduce affinity for ADP, thereby promoting an activated state and sensitizing the NLR.

In a reverse approach, Huang et al. [27], generated a bioengineered version of the tomato Sw-5b NLR to confer immunity towards resistance-breaking isolates of *tomato spotted wilt virus* (TSWV). First, a sensitizing mutation in the N-terminal part of the Sw-5b LRR was introduced [28], followed by random mutagenesis of the N-terminal extension of this NLR which is involved in effector recognition and is also known as the Solanaceae domain [27]. One of the resulting Sw-5b mutants can confer full immunity towards both previously resistance-breaking isolates of TSWV as well as the original TSWV strain [27].

In the case of R3a, two independent mutations in the CC or NB-ARC domain confer recognition of an immune-evading allele of the oomycete *P. infestans* Avr3a

[21]. Transferring the amino acid substitution found in the R3a CC domain to the tomato NLR I-2, a homolog of R3a which confers resistance towards strains of the fungus *Fusarium oxysporum* f. sp. *lycopersici* expressing the effector *Fo*Avr2 [29], resulted in an I-2 variant with an enhanced recognition spectrum [30]. I-2 weakly recognizes the *P. infestans* effector Avr3a, and the sensitized I-2 could respond towards immune-evading alleles of Avr3a as well as immune-evading alleles of *Fo*Avr2 [30]. This indicates that some sensitizing mutation can be transferred between NLRs to enhance recognition spectra.

Principle 3: mutating and swapping NLR "integrated" domains results in altered recognition

In some NLRs, effector-recognition is mediated by non-canonical "integrated" domains (IDs) [31]. These integrated domains are thought to be derived from effector-targeted disease susceptibility genes [32,33]. Given that these integrations appear to have happened repeatedly across the plant NLR phylogeny [6] and that these IDs are required for effector recognition, ID engineering or ID swapping is expected to alter the disease resistance spectra. Currently, two different NLR-IDs, both originating from rice, have been used as scaffolds to generate novel and enhanced disease resistance spectra: 1) Pia-2 (also known as RGA5), which is required for immunity towards AVR-Pia or AVR1-CO39 expressing strains of the rice blast pathogen *Magnaporthe oryzae* [34,35], and 2) different alleles of Pik-1, which are required for immunity against *M. oryzae* strains expressing alleles of AVR-Pik or AVR-Mgk1 [36,37]. Both Pia-2 and Pik-1 contain a heavy-metal-associated (HMA) domain: integrated either C-terminal of the LRR in the case of Pia-2, or between the CC and NB-ARC domain in the case of Pik-1 [34,35]. In addition, both the C-terminal HMA integration of Pia-2 [35,38,39], as well as the central HMA integration of Pik-1 are directly involved in effector binding [40,41].

Structure-guided mutagenesis of the Pia-2 HMA resulted in two mutants recognizing the *M. oryzae* effector AVR-PikD or AVR-Pib, respectively [42,43] (Figure 3a). In the case of AVR-PikD, recognition did not translate to enhanced immunity towards *M. oryzae* [42]. By contrast, AVR-Pib recognition by the engineered Pia-2 did result in immunity towards AVR-Pib expressing *M. oryzae* strains, but at the expense of AVR-Pia recognition [43]. Similarly, mutating the integrated HMA of the Pik-1 allele Pikp-1 resulted in an expanded recognition spectrum towards AVR-Pik alleles [44] (Figure 3b). Furthermore, swapping out the Pikp-1 HMA domain for a HMA domain which was found to bind all known AVR-Pik variants [45], and which was further engineered to prevent autoactivity, resulted in immunity against *M. oryzae* strains carrying these AVR-Pik alleles [46] (Figure 3b). Finally, a study from our lab recently showed that the integrated HMA domain of the Pik-1 allele Pikm-1 can be swapped for structurally unrelated "nanobodies" [47] (Figure 3c). Nanobodies are the antigen binding fragment of heavy chain only antibodies derived from camelids. We showed that integration of nanobodies binding fluorescent proteins could provide immunity against engineered *potato virus X* (PVX) strains expressing these fluorescent proteins [47]. Therefore, although the exact mechanism by which effector-binding to integrated domains activates NLR-IDs remains unknown, it is possible to mutate and even swap out IDs to enhance affinity towards novel effectors to generate novel immune resistance spectra.

Principle 4: synthetic logic gates can be designed to confer new recognition specificities

In a special case of NLR engineering, Wang et al. [48], generated a gene circuit in which an autoactive version of the Arabidopsis TIR-NLR pair RESISTANT TO RALSTONIA SOLANACEARUM 1 (RRS1)/RESISTANT TO P. SYRINGAE 4 (RPS4) is suppressed by a second non-autoactive RRS1. Conditional degradation of this second non-autoactive RRS1 copy is predicted to release the autoactive RRS1/RPS4 pair to trigger immune signalling. In order to achieve this, the second non-autoactive copy was mutated to prevent interaction with RPS4, and fused to the Arabidopsis GATA TRANSCRIPTION FACTOR 18 (GATA18) domain [48]. This GATA domain is targeted for ubiquitin-independent proteasomal degradation by the phytoplasma effector SAP05 [49]. Co-expression of all components in tobacco results in a specific hypersensitive cell-death which depends on SAP05. However, transgenic Arabidopsis lines expressing the engineered NLRs were not fully resistant towards phytoplasma infection [48].

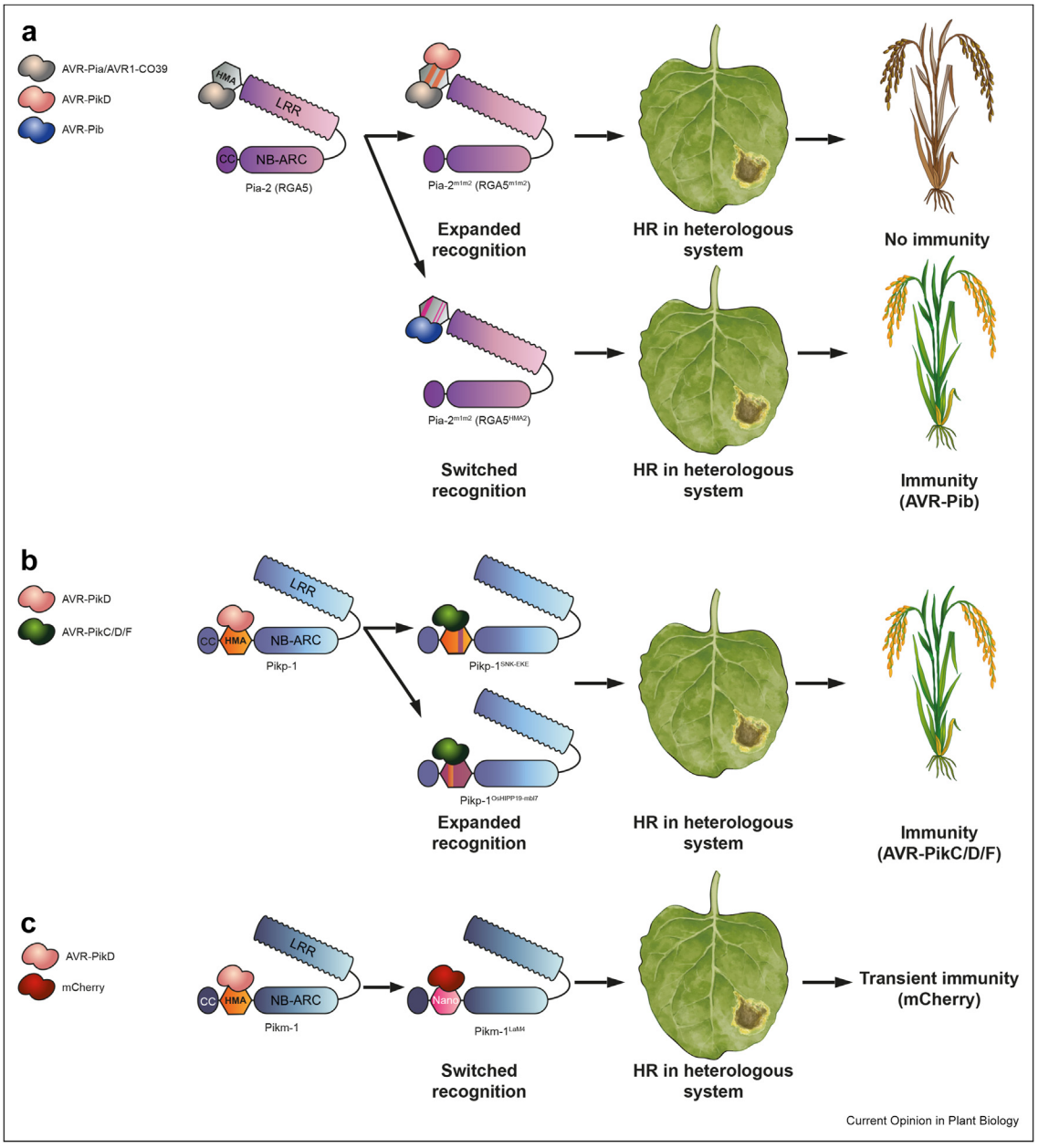
This approach of conditional de-repression of autoactive immune receptors could be extended towards other systems with the limitation that the current iteration requires extensive protein engineering and needs to be transformed into a genetic background that does not already carry a functional copy of the original immune receptors.

Principle 5: decoy engineering can alter effector recognition by NLRs

The activity of certain NLRs can be altered by mutating their host protein co-factors. These host proteins are conceptually defined as guardees or decoys, and immune receptors recognize effector-binding or effector-induced enzymatic modification of these proteins [50].

This guard/decoy model implies that decoy engineering could provide an effective way to engineer novel disease resistance specificities. Indeed, modifying the *Pseudomonas syringae* protease effector AvrPphB cleavage site in

Figure 3



Mutations and swaps of NLR-integrated domains result in altered immune spectra. **a**) Structure-guided mutation of Pia-2 resulted in the engineered Pia-2^{m1m2} (Pia-$2^{E1029A/I1030L/T1031V/E1033D/D1034L/K1035R/R1037K/L1038I/V1039E/M1065Q/E1067S/L1068Q}$) which confers recognition of the *M. oryzae* effector AVR-PikD [42], and Pia-2^{HMA2} (Pia-$2^{G1009D/S1027V/K1071E/K1073E/K1080E/K1081E/K1085E/K1086E}$) which confers immunity towards strains of *M. oryzae* expressing the AVR-Pib effector at the expense of AVR-Pia recognition [43]. **b)** Either structure-guided mutation of Pikp-1 resulting in the engineered Pikp-$1^{SNK\text{-}EKE}$ (Pikp-$1^{S258E/N261K/K262E}$), or swapping the integrated domain (ID) of Pikp-1 for the related rice heavy metal associated isoprenylated plant protein 19 (*Os*HIPP19) HMA domain which was engineered to prevent autoactivation (Pikp-$1^{OsHIPP19/mbl7}$), results in immunity towards *M. oryzae* strains expressing either AVR-PikC, AVR-PikD, or AVR-PikF [46]. **c**) Swapping the integrated HMA of Pikm-1 for structurally unrelated nanobodies results in immunity towards engineered PVX strains expressing either GFP or mCherry [47].

AVRPPHB SUSCEPTIBLE 1 (PBS1)—a receptor-like cytoplasmic kinase which is guarded by the Arabidopsis NLR RESISTANT TO P. SYRINGAE 5 (RPS5)—is sufficient to engineer novel disease resistance specificities [51]. Replacing the AvrPphB cleavage site with the cleavage site of the *P. syringae* effector AvrRpt2 was sufficient to generate resistant plants towards *P. syringae* strains carrying AvrRpt2 [51]. A similar approach was used to engineer resistance to *turnip mosaic virus* (TuMV) in Arabidopsis using the TuMV NIa protease cleavage site

[51,52]. Because AvrPphB-dependent modification of PBS1 homologs is recognized in many plant species by unrelated NLRs [53], it is possible to engineer these PBS1 homologs in a similar manner even without knowing the identity of the guarding NLRs. For example, engineering *soybean mosaic virus* (SMV) resistance in soybean [52,54], or *potato virus Y* (PVY) resistance in potato [55], is possible by introducing the respective viral NIa protease cleavage sites into PBS1 homologs from these species [52,54,55].

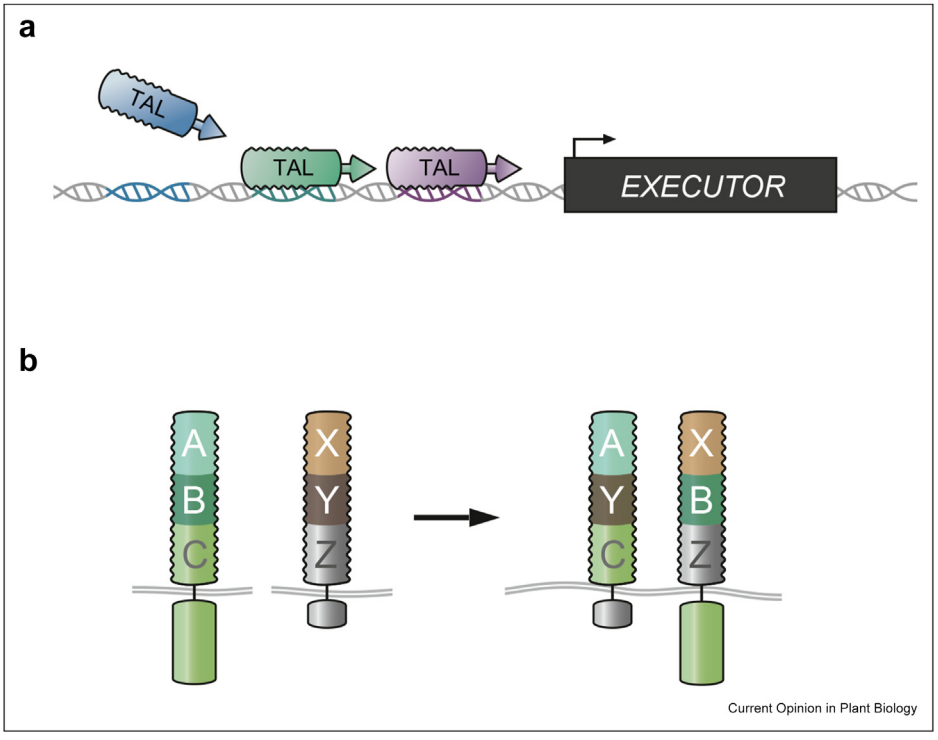
Engineering PBS1 has potential limitations due to the nature of the amino acids as well as the length of the cleavage site. Indeed, adding extra residues within the PBS1 cleavage site results in autoactivation [56], and editing PBS1 homologs within a native context can result in the loss of AvrPphB recognition. Furthermore, effective immunity against TuMV also requires *PBS1* expression from a strong constitutive promoter [52], indicating that gene editing may result in recognition without effective disease resistance. However, given that indirect recognition of pathogen effectors by NLRs seems to be a common mechanism [2], decoy engineering could potentially be extended to engineering other NLRs recognition mechanisms.

There is more to plant immunity than NLRs

Finally, even though NLRs are the main class of characterized plant immune receptors, for most pathogens there is more to plant immunity than just NLRs. Other classes of immune receptor also provide useful scaffolds for bioengineering. While they are not strictly immune receptors, bioengineered executor genes can provide novel disease resistance to *Xanthomonas* species translocating transcription activator-like (TAL) effectors [57] (Figure 4a). The expression of these executor genes is activated by binding of TAL effectors to specific promoter elements within the executor genes, thereby resulting in activation of the hypersensitive cell death response [58]. Fusing multiple TAL effector DNA binding elements together is sufficient to bioengineer made-to-order executor genes [57] (Figure 4a).

In addition to intracellular immunity, the plant cell-surface and extracellular space constitute a main host-pathogen interaction interface. Cell-surface receptors are involved in direct and indirect recognition of pathogens at this interface. In plants, cell-surface receptors have been bioengineered by 1) swapping the intracellular signalling module between plant cell-surface

Figure 4



Bioengineering other classes of disease resistance genes for enhanced immunity. Non-NLR immune systems have been engineered by **a)** shuffling of DNA binding elements to engineer novel executor genes providing disease resistance against various *Xanthomonas* species expressing specific TAL effectors, **b)** swap residues and domains between cell-surface receptors to generate cell-surface receptors with altered downstream signalling or swapped ligand recognition.

receptors to redirect signalling downstream of recognition [59–63], or 2) or by domain swapping and targeted mutagenesis of extracellular ligand-binding domains to exchange the recognition specificities of these cell-surface receptors between homologs [64–67] (Figure 4b). The exact mechanism by which mutations in the extracellular ligand-binding domains result in activation of these immune receptors by different ligands remains to be shown and could result in additional design principles.

Conclusion

In conclusion, we describe five emerging principles in the design and application of bioengineered plant NLR immune receptors. We anticipate that delineating these principles may facilitate the understanding of the function of these NLRs, as well as drive further engineering of made-to-order plant immune receptors. It is expected that more design principles will emerge as research continues, or that increased understanding of the mechanism by which some mutations function will result in them being categorized under different principles. Additionally, we anticipate that other classes of plant immune receptors, such as cell-surface receptors, will serve as useful scaffolds for bioengineering novel disease resistance specificities. Finally, in addition to receptor-based bioengineering, there are many different approaches to enhance disease resistance which do not rely on receptors (see for example Dangl et al., 2013 [68]). Generating durable disease resistance will likely rely on a combination of natural *R* genes, bioengineered receptors, and engineered non-receptor-based approaches. Combining multiple receptor and non-receptor based resistance genes in a single "stack" has already been proven to be feasible [69–71]. While prior knowledge is not always required to design bioengineered receptors, our growing understanding of the recognition and activation mechanisms of immune receptors enables rational engineering approaches.

Funding

This work has been supported by the Gatsby Charitable Foundation (SK), Biotechnology and Biological Sciences Research Council (BBSRC) (SK), Rijk Zwaan Netherlands (HP, SK), Groupe Limagrain (CM, SK), and BASF Plant Science (JK, SK). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

Clemence Marchal: Conceptualization, Writing - Review & Editing, Visualization. Hsuan Pai: Visualization. Sophien Kamoun: Writing - Review & Editing, Project administration, Funding acquisition. Jiorgos Kourelis: Conceptualization, Investigation, Data curation, Writing - Original Draft, Writing - Review & Editing, Visualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships that may be considered as potential competing interests: Sophien Kamoun, Jiorgos Kourelis reports financial support was provided by BASF Plant Science GmbH. Clemence Marchal, Sophien Kamoun reports financial support was provided by Groupe Limagrain. Hsuan Pai, Sophien Kamoun reports financial support was provided by Rijk Zwaan Netherlands. Jiorgos Kourelis, Clemence Marchal, Sophien Kamoun has patent pending to NA.

Data availability

No data was used for the research described in the article.

Acknowledgments

JK would like to thank Alexander Förderer for discussions which shaped some of the ideas presented in this review. We would also like to thank members of the Kamoun lab as well as the wider TSL community for discussions and ideas which contributed to this work. We also thank Bodhidharma for their guidance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbi.2022.102311.

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