Molecular engineering of plant immune receptors for tailored crop disease resistance

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Molecular engineering of plant immune receptors for tailored crop disease resistance

Lila Cadiou[1], Francois Brunisholz[1], Stella Cesari and Thomas Kroj

Abstract

The specific recognition of pathogen effectors by intracellular nucleotide-binding and leucine-rich repeat domain receptors (NLRs) is an important component of plant immunity. Creating NLRs with new bespoke recognition specificities is a major goal in molecular plant pathology as it promises to provide unlimited resources for the resistance of crops against diseases. Recent breakthrough discoveries on the structure and molecular activity of NLRs begin to enable their knowledge-guided molecular engineering. First, studies succeeded to extend or change effector recognition specificities by modifying, in a structure-guided manner, the NLR domains that directly bind effectors. By modifying the LRR domain of the singleton NLR Sr35 or the unconventional decoy domains of the helper NLRs RGA5 or Pik-1, receptors that detected other or additional effectors were created.

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Keywords

Disease resistance, Plant immunity, Immune receptor, NLR, Nucleotide-binding and leucine-rich repeat domain protein, Resistance protein.

Introduction

The natural immunity of crops to diseases is fundamental for their protection from disease but also constantly threatened by the outstanding capacity of pathogen populations to adapt [1–3]. A frequent mechanism for pathogens is to evade detection by plant immune receptors that activate potent defense responses [4]. These receptors form the prevalent class of plant resistance genes and are extensively used to breed disease-resistant varieties [5]. Generating immune receptors with tailored recognition specificities by molecular engineering could provide powerful new solutions for sustainable plant protection and, thereby, help to counter the tremendous adaptive potential of plant pathogens [2,6,7]. In this article, we summarize recent breakthroughs in research on nucleotide-binding and leucine-rich repeat domain receptors (NLRs) and present the latest work on their knowledge-guided engineering, which offers new and concrete opportunities for the creation of disease-resistant crops.

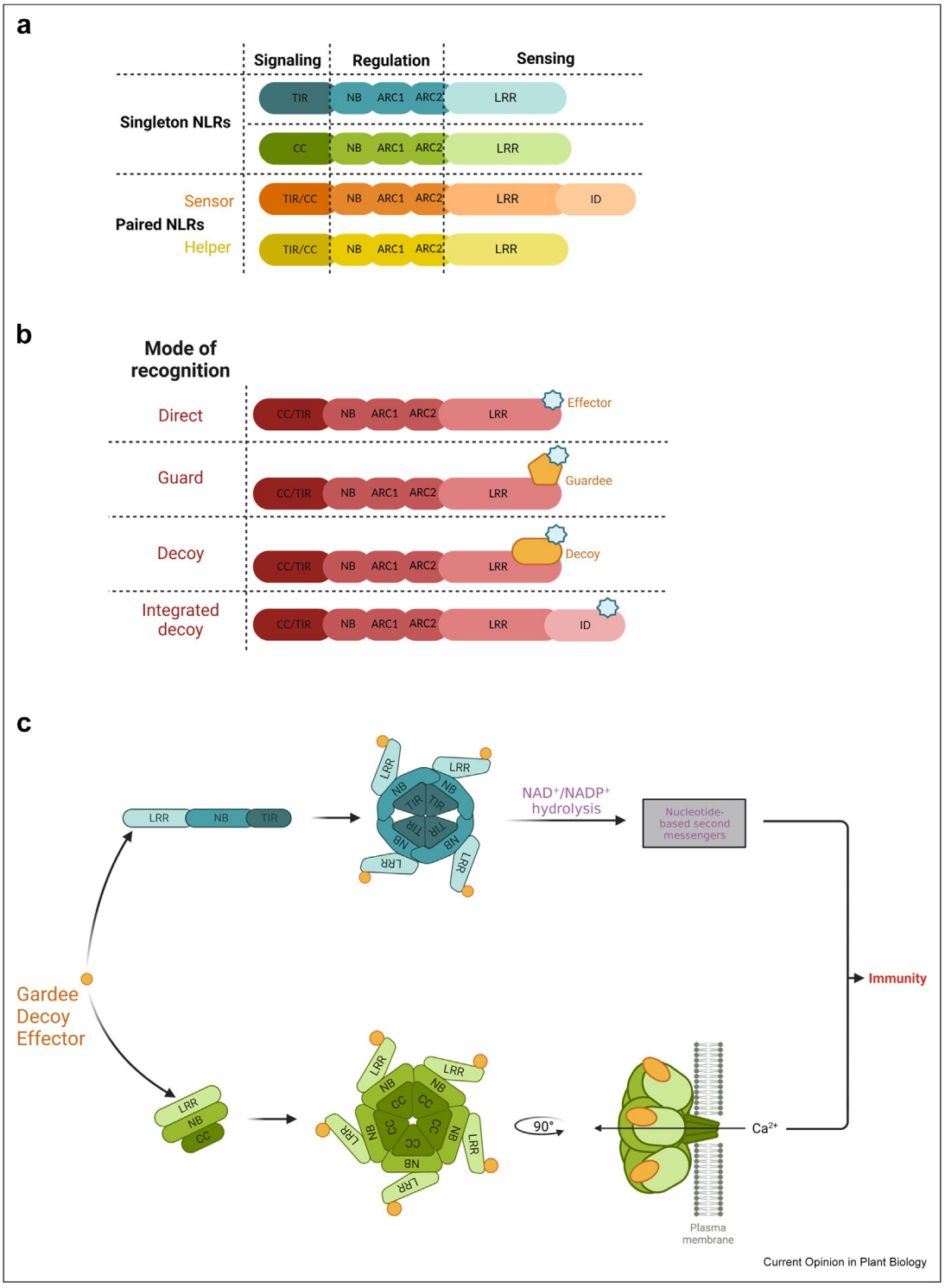
NLRs – the guardians of plant health

NLRs are the major class of immune receptors in plants [5]. They recognize pathogen-effector proteins inside the host cell and initiate signaling that triggers immune responses [8,9]. NLRs are defined by a conserved structure comprising three canonical domains: a variable N-terminal domain that is, in most cases, either a coiled-coil (CC) or a toll/interleukin-1 receptor (TIR) domain, a C-terminal leucine-rich repeat (LRR) domain, and a central nucleotide-binding adaptor shared by APAF-1, resistance proteins, and CED-4 (NB-ARC) domain. The NB-ARC domain is further subdivided into an NBD, a helix domain (HD, also named ARC1), and a winged helix domain (WHD, also named ARC2) (Figure 1a) [8,9]. Effector detection is mainly mediated by the LRR domain that either physically binds the effector or detects effector-induced modifications of host proteins (Figure 1b). The NB-ARC acts as a molecular switch and transduces this molecular recognition to the N-terminal domain, which activates signaling.

3D structures shed light on the molecular function of NLRs

The recent elucidation of the structures of the NLRs ZAR1, RPP1, and ROQ1 from *Arabidopsis thaliana* and Sr35 from wheat in their active conformation and for ZAR1 also in its inactive form has provided groundbreaking insights into the biochemical functions of NLRs (Figure 1c) [10–15]. These studies illustrated how the central NB-ARC domain controls the activity of the receptor depending on its nucleotide-binding status. ADP binding stabilizes the closed, inactive

Figure 1



Structure and function of NLR immune receptors, (**a)** NLRs are composed of three canonical domains: An N-terminal signaling domain that is mostly a toll/interleukin-1 receptor (TIR) or coiled-coil (CC) domain, a central nucleotide-binding adaptor shared by APAF-1, resistance proteins, and CED-4 (NB-ARC) domain that regulates the activity of the receptor, and a C-terminal leucine-rich repeat (LRR) domain that is crucial for signal detection. Some NLRs carry, in addition, non-canonical integrated domains (IDs) that are highly variable and can be located at various positions, for example, before the NB-ARC or after the LRR. Some NLRs function in pairs where one NLR acts as a sensor (sNLR) that detects the effector and always carries an ID and one as a helper (hNLR) that activates immune signaling. Domains are not drawn to scale. (**b)** NLRs can recognize effectors in multiple ways. Directly, in a receptor-ligand type of interaction where the effector binds directly the NLR, mostly through the LRR domain, or indirectly, where the modification of a plant protein by an effector is sensed or a complex between the effector and a plant protein is recognized. The plant protein can be either a target protein of the effector

conformation of the receptor [10]. Signal recognition leads to a steric clash with the NBD that induces conformational changes in the NB-ARC and binding of ATP, which stabilizes the active, open conformation of the protein [10,11,14,15]. In this activated conformation, NLRs assemble through intermolecular interactions into a homomeric supramolecular signaling-competent complex called the resistosome (Figure 1c). The CC-NLRs ZAR1 and Sr35 form resistosomes composed of five subunits that act as cation channels [14,16]. The ion pore itself is formed by the N-terminal amphipathic alpha helix of the CC domain that is reconfigured and exposed to form a funnel-shaped structure that inserts into the plasma membrane and is selective for Ca^{2+} ions.

The TIR-NLRs RPP1 and ROQ1 form tetrameric resistosomes where the TIR domains form two asymmetric dimers (Figure 1c) [12,13]. TIR dimers possess enzymatic activity on $NAD(P)^{+}$, single-stranded RNA, and double-stranded DNA, as well as catalyze the formation of nucleotide-derived compounds that act as second messengers in the activation of immunity, which involves downstream helper NLRs [17–21]. These helper NLRs possess a specific type of CC domains and activate signaling in a similar manner than CNLs [22].

The resistosome structures also revealed shared mechanisms in effector recognition. Indeed, a similar region at the end of the LRR domain (the ascending lateral side of the LRR horseshoe structure) binds the effectors or, in the case of ZAR1, the decoy kinase RKS1 that senses effector activity [10–15]. These binding and recognition events create the steric conflict with the NBD, which triggers the rotation of the NBD and the HD and re-arrangement of the NB-ARC domain in its active ATP-binding configuration.

NLR resistosome structures pave the way toward knowledge-guided LRR engineering

The improved understanding of NLR function provides new perspectives for the creation of NLRs with novel high-affinity binding sites for new effectors or effector-modified host proteins by molecular engineering. Indeed, the NLR resistosome structures highlight opportunities in engineering the common effector or decoy-binding region at the end of the LRR, as well as show that the binding of the new ligand has to produce a steric clash with the NBD sufficient to induce NB-ARC domain reconfiguration and NLR activation. The resolution of the Sr35 resistosome structure allowed performing such a structure-informed approach for the first time [14]. Indeed, the comparison of the effector-binding interface of Sr35 with the corresponding LRR region of two different Sr35 homologs of unknown resistance function (~86% overall amino acid sequence identity to Sr35) identified a limited number of polymorphisms in key effector-binding residues. Substitution of these residues in these Sr35 homologs enabled recognition of the effector AvrSr35 in transient expression assays [14]. It remains to be demonstrated that these designer NLRs provide stem rust resistance in whole plant assays with stable transgenic lines.

In some NLRs, it will certainly be necessary to modify additional sites located outside the main effector and decoy-binding region in the LRR to create new immune receptors. Binding of the effector XopQ by ROQ1 relies, for instance, on multiple sites in the LRR and intimate binding to a post-LRR domain of ROQ that is present in 50% of all TNLs [13,23]. Engineering of such TNLs will, therefore, presumably require mutations at multiple sites in the LRR and the post-LRR domain.

The integrated domains of sensor NLRs are crucial for effector detection and promising targets for NLR engineering

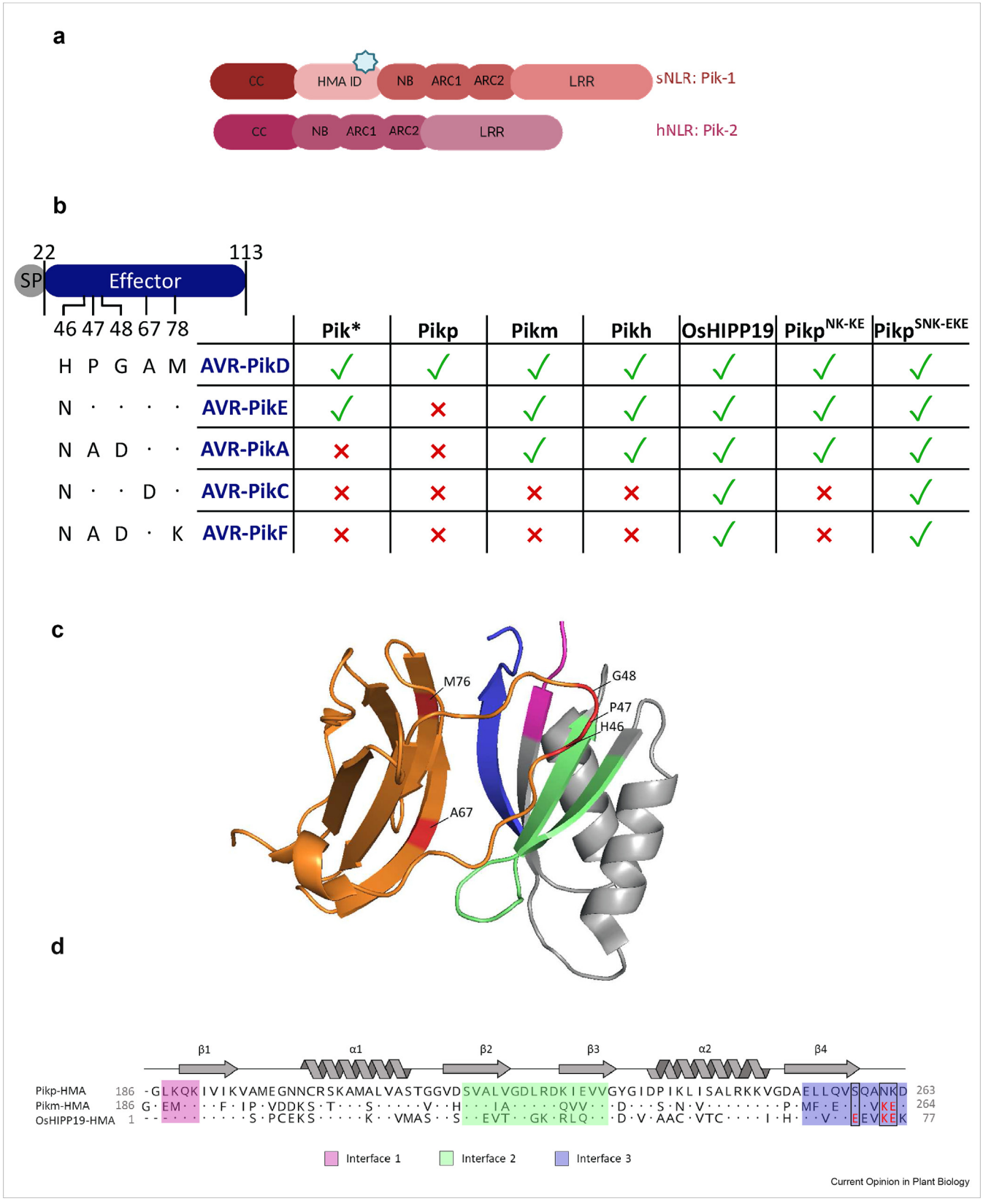
In addition to singleton NLRs, plants possess NLR pairs composed of one sensor and one helper NLR (sNLR and hNLR) that are both required for disease resistance (Figure 1a) [24]. Such sNLRs from pairs harbor non-canonical integrated domains (IDs) that mimic effector targets and bind or are modified by effectors (Figure 1b). In three model systems, the TIR-NLR pair RRS1/RPS4 from *A. thaliana* and the CC-NLR pairs RGA4/RGA5 and Pik-1/Pik-2 from rice, the central role of the ID in effector recognition has been established, and its interaction with effectors was studied at the structural level [24]. This detailed mechanistic and structural insight was used for sNLR engineering in the Pik-1/Pik-2 and RGA4/RGA5 systems. These studies, discussed in the following, demonstrate that the creation of plant immune receptors with new recognition properties is feasible and offer exciting prospects for the design of immune receptors with bespoke effector recognition specificities.

Pik-1 engineering – inspired by nature to keep pace with the fast evolution of the blast fungus toward escape from the detection of the AVR-Pik effector

The NLR pair Pik-1/Pik-2 detects the effector AVR-Pik from the fungal pathogen *Pyricularia oryzae* (syn. *Magnaporthe oryzae*) that causes the devastating blast

and is then called a guardee or it can be a mimic of a target protein that has no other function than trapping effectors and is then called a decoy. NLR-IDs function by an integrated decoy mechanism, where the effector is detected through its binding to the ID or by the modification, it causes on the ID. **(c)** NLRs multimerize upon effector recognition into a signaling-competent resistosome that is composed of four TIR-NLR or five CC-NLR subunits. The pentameric CC-NLR resistosome integrates into the plasma membrane through the funnel-shaped structure formed by the N-terminal alpha helix of the CCs and mediates influx of cations with specificity for Ca^{2+} ions that act as second messengers in immune activation. The asymmetric TIR dimers in the tetrameric TIR-NLR resistosome catalyze NAD+/NADP + hydrolysis which results in the production of nucleotide-based second messengers.

Figure 2



The HMA domain of Pik-1 binds AVR-Pik in an allele-specific manner that can be modified by molecular engineering. (**a**) The rice sNLR/hNLR pair Pik-1/Pik2 detects the effector AvrPik of the blast fungus *P. oryzae*. The HMA ID of Pik-1 that is located between its CC and NB-ARC domains directly binds

disease on cereals (Figure 2a) [25—27]. This recognition is allele specific as there exist six alleles of AVR-Pik (A to F) and numerous functional alleles of Pik-1/Pik-2, with different recognition specificities (Figure 2b) [28—30]. The p allele of Pik-1/Pik-2 detects, for instance, only AVR-PikD, while the m allele has a broader recognition spectrum and detects the D, A, and E alleles. AVR-PikC and F are not detected by any variant of Pik-1/Pik-2.

The sNLR Pik-1 binds the effector through its integrated heavy metal-associated (HMA) domain, which is critical for detection and involves three different interfaces, all located on the β-sheet of the HMA (Figure 2c) [28,31] The HMA domains of Pikp-1 and Pikm-1 differ in key effector-binding residues, which results in varying contributions of the interfaces to effector binding [32]. Indeed, the HMA interface 3 of Pikm-1 contributes strongly to effector binding and enables detection of AVR-PikA, D, and E, while in Pikp-1 HMA, its contribution is only very weak. Pikp-1 can, therefore, only detect AVR-PikD that is strongly bound through interface 2 of the HMA, while the other effector alleles are not.

This insight into effector/ID interactions allowed to transfer the broad binding and recognition spectrum of Pikm-1 to Pikp-1 by replacing two key residues in the binding interface 3 of the Pikp-1 HMA (Figure 2d) [33]. This achievement provided a proof of concept for the transfer of effector recognition specificities between NLR alleles by ID engineering.

Further extension of the recognition spectrum of Pikp-1 to the C and F alleles of the effector was achieved by exploiting the absence of allele specificity in the binding of AVR-Pik to small HMA proteins (sHMAs), a huge plant gene family of unknown function and putative virulence targets of AVR-Pik [34—37]. By replacing the HMA of Pikp-1 by that of the sHMA OsHIPP19 or by exchanging only three specific residues between the two HMAs, it was possible to create Pikp-1 variants that bind all AVR-Pik alleles, recognize them in the heterologous *N. benthamiana* model plant, and confer resistance to corresponding *P. oryzae* isolates in transgenic rice plants (Figure 2d) [38].

This pioneering work provides two groundbreaking advances. It replaces for the first time the ID of an sNLR by an effector target protein to confer an extended resistance spectrum, and it modifies in a structure-informed manner an ID to detect effector alleles not detected by the naturally occurring receptor.

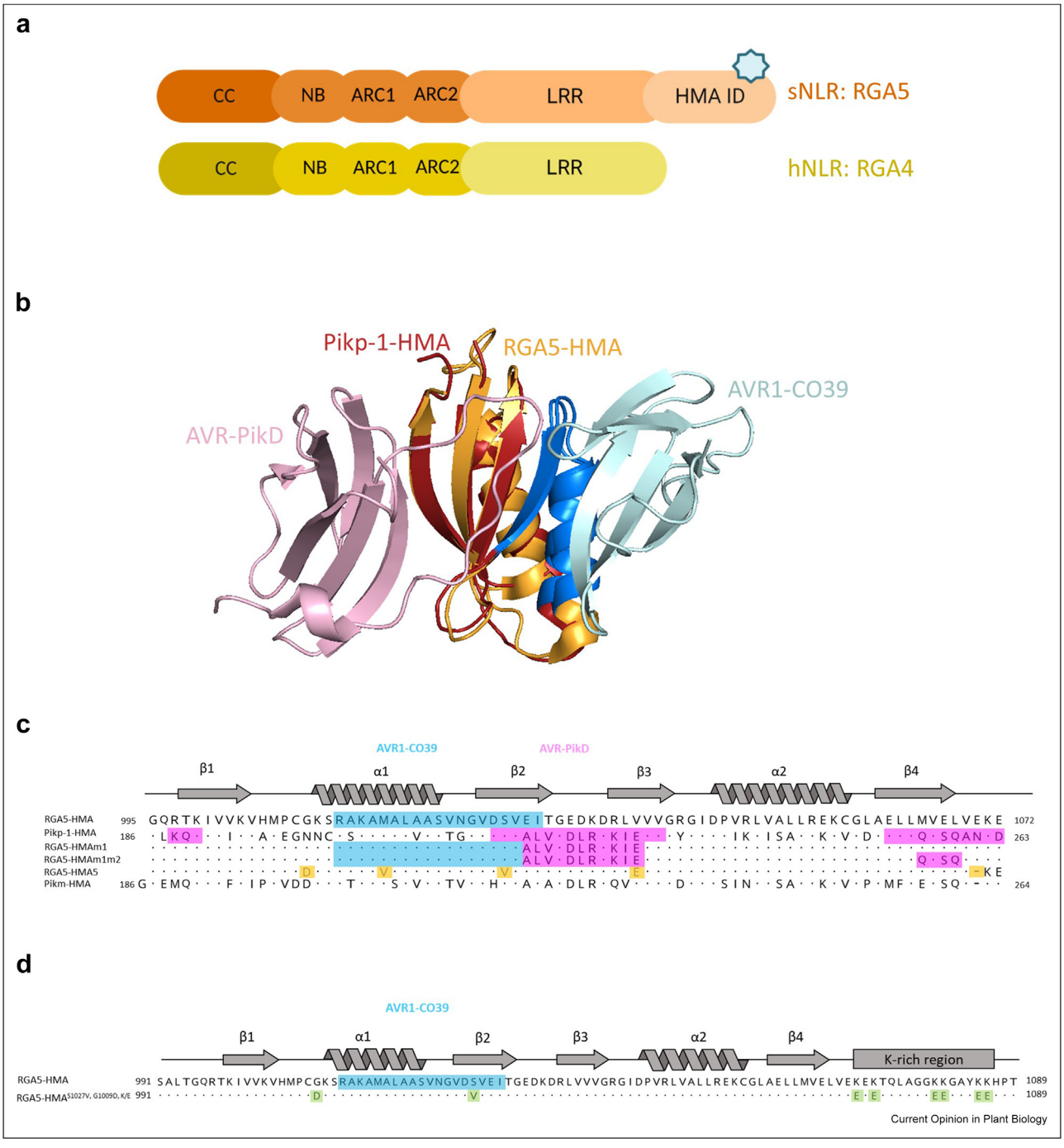
RGA5 engineering – different surfaces of the HMA ID can be reshaped to create new effector recognition specificities

The rice hNLR/sNLR pair RGA4/RGA5 detects the *P. oryzae* effectors AVR-Pia and AVR1-CO39 that are sequence unrelated but have the same structure, characteristic of the MAX effector family, which also comprises AVR-Pik (Figure 3a) [39—43]. Like Pik-1, RGA5 has an HMA ID that binds AVR-Pia and AVR1-CO39 and is crucial for their recognition. Despite these apparent similarities, the effector/ID complexes formed in the RGA5 and Pik-1 systems are completely different as they involve different surfaces of the effectors and the HMAs, which are located in both cases almost on opposite sides of the molecules (Figure 3b) [44,45].

Based on the detailed knowledge of the structures of the effector/HMA ID complexes, a high-affinity binding site for AVR-PikD was generated in the HMA of RGA5 [46]. This was achieved by swapping into the HMA of RGA5 the polymorphic residues of the AVR-PikD binding interface 2 of the Pikp-1 HMA that is crucial for effector binding (Figure 3c). In the heterologous *N. benthamiana* system, these changes enable recognition of AVR-PikD by engineered RGA5 and do not impair detection of AVR-Pia. However, in rice plants, the HMA-engineered RGA5 variants only conferred resistance to *P. oryzae* isolates expressing *AVR-Pia* or *AVR1-CO39*, but not to those carrying *AVR-PikD* [46]. We hypothesize that the position of the new AVR-PikD binding site does not allow for receptor activation or that proper activation of immunity requires that effectors interact with additional motifs of RGA5 outside of the HMA.

AVR-Pik, which is crucial for effector detection and immunity. (**b)** AVR-Pik alleles differ at five polymorphic sites located in the mature protein produced by cleavage of the signal peptide (SP). They all bind to the sHMA protein HIPP19 and are differentially recognized by natural Pik-1 alleles and the engineered variants Pikp$^{NK\text{-}KE}$ and Pikp$^{SNK\text{-}EKE}$ (details on these variants are in panel D). Detection *in planta* by the Pik-1 full-length receptor is correlated and explained with the binding to the HMA ID. Critical polymorphic residues in AVR-Pik are either involved in binding the HMA like histidine 46 (details in panel C) or act by their disruptive effect on binding like aspartate 67 or lysine 78 in, respectively, AVR-PikC and F. (**c)** Three-dimensional structure of the AVR-PikD/Pikp-1 HMA complex (PDB 6G10) with the three different binding interfaces highlighted in different colors (same colors as in D) and AVR-PikD in orange with polymorphic residues in red. Histidine 46 in AVR-PikD (c.f panel D) interacts directly with residues in interface 2 of the Pikp-1 HMA domain. When it is replaced, the binding to interface 2 is drastically attenuated, which results in weak overall binding and lack of detection. In other Pik-1 alleles than Pikp-1, this weak contribution of interface 2 is compensated by polymorphisms located at the end of the HMA domain that result in strong bonding between interface 3 and the effector. (**d)** Sequence polymorphism in the HMA domains of Pikp-1, Pikm-1, and HIPP19. The replacement of the residues highlighted by black boxes, and red color in the HMA of Pikp-1 extends the effector recognition spectrum of the receptor. The mutant variant Pikp-1$^{NK\text{-}KE}$ binds and detects AVR-PikA and E in addition to AVR-PikD because the replacement of specific asparagine and lysine residues in interface 3 by, respectively, lysine and glutamate strongly increases the binding strength. Pikp-1$^{SNK\text{-}EKE}$ recognizes, in addition, AVR-PikC and F because the additional glutamate residue from interface 3 of HIPP19 engages strong bonding with all AVR-Pik alleles. This is necessary because the critical polymorphic residues of AVR-PikC and F (respectively, positions 67 and 78) negatively interfere with binding to the Pik HMA domain.

Figure 3



The RGA5 HMA domain can be modified to bind AVR-PikD and AvrPib and detect them in a whole plant context. (**a)** Overlay of the three-dimensional structures of the AVR-PikD/Pikp-1 HMA and the AVR1-CO39/HMA complexes (respectively, PDB: 6G10 and 5ZNG). While the RGA5 HMA interacts through its α1 helix and β2 strand (labeled in blue) with the β1 and β2 of AVR1-CO39, the Pikp-1 HMA binds through an extended surface covering large parts of its β-sheet to the N-terminal extension and the β2 and β3 strands of AVR-PikD. These fundamental differences suggest that the detection of MAX effectors by HMA IDs has evolved independently in RGA5 and Pik-1. (**b)** Sequence polymorphism in the HMA domains of RGA5, Pikp-1, and Pikm-1 and changes introduced into the RGA5 HMA domain to create an AVR-PikD detection specificity. The swap of the AVR-Pik binding interface 2 of Pikp-1 HMA into RGA5 HMA produces a high-affinity binding site for this new effector without affecting binding of AVR-Pia and AVR1-CO39 (RGA5-HMAm1). Introduction of additional residues from binding interface 3 leads to a further but slight increase in binding (RGA5-HMAm1m2). The corresponding full-length RGA5 receptors detect all three MAX effectors in the *N. benthamiana* model but only AVR-Pia and AVR1-CO39 in transgenic rice. In an independent study, AVR-PikD binding and recognition was conferred to the HMA domain of RGA5 by combining structure-informed changes in the AVR1-CO39 binding interface with point mutations in the β strands 3 and 4 (RGA5-HMA5). These point mutations introduce one important residue from binding interface 2 of Pikm-1 HMA and reposition the critical lysine and glutamate residues at the end of binding interface 3 as in Pikm-1 HMA. (c) Modifications in

In another attempt to confer AVR-PikD recognition to RGA5, the AVR1-CO39 and AVR-Pia-binding interface of RGA5 HMA was modified to interact with AVR-PikD; in addition, two key AVR-Pik binding residues from the interface 2 and 3 of the HMA domain of Pikm-1 were introduced (Figure 3c) [47]. The resulting RGA5 mutant supports AVR-PikD binding, recognition of the new effector in the heterologous *N. benthamiana* system, and, importantly, resistance of rice to *P. oryzae* expressing AVR-PikD. However, the mechanism underlying the detection of the new effector remains unclear since it is not known to which of the two effector-binding interfaces of the modified HMA domain AVR-PikD binds.

In a third study, RGA5 was engineered to bind and recognize AvrPib, another MAX effector of *P. oryzae*, which has no sequence similarity to AVR1-CO39, AVR-Pia, or AVR-Pik [48]. Modification of the AVR-Pia and AVR1-CO39-binding interface of RGA5 HMA enabled its interaction with AVR-Pib, but failed to confer effector recognition. However, the additional mutation of a stretch of basic residues, located just after the HMA, resulted in AvrPib recognition and immunity in transgenic rice lines expressing the engineered receptor (Figure 3d) [48]. The underlying mechanism remains unknown, but it was proposed that the additional mutations enhance the binding of the new effector. This example highlights that, in some cases, it can be necessary to modify sequences outside the ID to create new functional immune receptors.

In conclusion, these studies on RGA5 have achieved for the first time the engineering of an NLR ID for the recognition of new sequence-unrelated effectors. However, these studies also highlight that our knowledge on the activation mechanisms of the RGA4/RGA5 receptor complex remains extremely limited, , that is, we do not know how effector binding is translated into immune receptor activation.

Perspectives for NLR research and engineering

Previous work had already demonstrated that swapping entire LRR domains between closely related NLR alleles, or exchanging polymorphic residues between them, permits to exchange effector detection specificity [49–52]. However, for Sr35, such an approach exploited for the first time structural insight into effector binding and resistosome formation and could thereby focus on an extremely limited number of residues [14]. This work provides the exciting perspective that the creation of resistance proteins through the structure-guided replacement of a limited number of residues by genome editing could soon become common practice and partially replace lengthy and tedious breeding for new resistance traits. A future challenge will be to create new recognition specificity in the effector-binding region of NLR LRRs and not only transfer it from already existing immune receptors. Prerequisites for such approaches are additional NLR resistosome structures to assist molecular engineering by structure modeling as well as efficient technologies to create and screen high numbers of NLR mutants, carrying sequence variations in the LRR domain, for effector binding and/or recognition.

The structure-informed modification of the IDs of sNLRs emerges as another highly promising approach for the engineering of disease resistance. Different studies prove its feasibility, and the next steps are more far-reaching modifications of the ID or their replacement by completely new effector-binding domains that may be effector target proteins or affinity probes. A proof of concept for such synthetic immune receptors is the demonstration that the replacement of the HMA domain of Pikp-1 by an anti-GFP or anti-mCherry nanobody generates sNLRs that detect these fluorescent proteins and confer resistance to viruses expressing them in transient assays and transgenic plants [53]. A major limitation for the future development of ID engineering is the lack of precise knowledge on the interactions of IDs with the rest of the sNLRs and of sNLR/hNLR interactions. Indeed, how effector binding influences the structure of the RGA5 or Pik-1 full-length proteins and which molecular events occur to allow downstream signaling by RGA4 or Pik-2, remain open questions. Further studies are needed to address these critical issues and enable in the future even more straightforward engineering of disease resistance involving sNLR/hNLR immune receptor complexes.

A general conclusion from current NLR engineering studies is that it is critical to analyze the function of promising engineered NLRs in stable transgenic lines. Transient assays are a powerful mean to test at high throughput mutant constructs for effector detection and immune activation. However, frequently, mutant variants that showed extended effector detection specificities in transient assays behaved as a transgene not as expected. Testing engineered NLRs for their capacity to confer enlarged or new resistance specificities in transgenic plants is, therefore, a critical experiment in this type of studies.

the HMA domain of RGA5 to permit detection of AvrPib. The combination of structure-informed changes in the AVR1-CO39-binding interface of the HMA domain together with the exchange of 6 lysine residues against glutamate in the basic sequence adjacent to the HMA conferred AvrPib binding to a RGA5-HMA$^{S1027V,\ G1009D,\ K/E}$ mutant fragment. An RGA5 variant with these changes in the context of the full-length receptor recognizes AvrPib in *N. benthamiana* cell death assays and confers together with RGA4 resistance in rice to *P. oryzae* isolates carrying AvrPib.

Transgenic lines are also critical to detect eventual deleterious phenotypes caused by engineered NLRs. Indeed, NLRs require precise regulation, and even weak constitutive activity of mutant alleles can have strong impact on plant physiology and yield. Therefore, careful phenotyping under various environmental conditions including field trials will be required to exclude that stronger immunity from tailored immune receptors comes under certain growth conditions with trade-offs and yield reduction. While NLR engineering has the potential to provide unlimited numbers of new, bespoke, and broad-spectrum immune receptors, it will not solve the problem of their limited durability. This issue will have to be addressed by their deployment strategies that should avoid the direct exposure of the new immune receptors to the enormous adaptive potential of pathogens. Powerful solutions are, for instance, multi-lines or variety mixtures that exploit the diversity and complementary of NLRs to prevent the rapid adaptation of pathogens to crop immunity and that have the potential to provide durable disease resistance [54,55]. Only in this context will NLR engineering find its true purpose. Therefore, there is an urgent need to foster the profound changes in legislation and the habits of major stakeholders, such as seeds companies and farmers, which are required to make this profound transition possible.

Authors' contribution

L.C, F.B., S.C., and T.K. wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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This study identifies together with reference 19 a missing links between TIR-NLR activation and the activation of immunity by downstream helper NLRs. It reports that activated TIR-NLRs produce pRib-AMP and pRib-ADP that bind to an EDS1-PAD4 complex. This triggers the association of the heterocomplex with the helper NLR ADR1 and activation of immune signaling.

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