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Review

The resistance awakens: Diversity at the DNA, RNA, and protein levels informs engineering of plant immune receptors from *Arabidopsis* to crops

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Abstract

Plants rely on germline-encoded, innate immune receptors to sense pathogens and initiate the defense response. The exponential increase in quality and quantity of genomes, RNA-seq datasets, and protein structures has underscored the incredible biodiversity of plant immunity. Arabidopsis continues to serve as a valuable model and theoretical foundation of our understanding of wild plant diversity of immune receptors, while expansion of study into agricultural crops has also revealed distinct evolutionary trajectories and challenges. Here, we provide the classical context for study of both intracellular nucleotide-binding, leucine-rich repeat receptors and surface-localized pattern recognition receptors at the levels of DNA sequences, transcriptional regulation, and protein structures. We then examine how recent technology has shaped our understanding of immune receptor evolution and informed our ability to efficiently engineer resistance. We summarize current literature and provide an outlook on how researchers take inspiration from natural diversity in bioengineering efforts for disease resistance from Arabidopsis and other model systems to crops.

Introduction

Like all organisms, plants can sense the presence of pathogens and induce immune responses. In the absence of adaptive immunity, plant innate immune receptors provide sufficient diversity at a population level to recognize rapidly evolving pathogens, including viruses, bacteria, nematodes, fungi, oomycetes, insects, and parasitic plants (Hamilton et al. 1990; Dangl and Jones 2001; Chisholm et al. 2006). Understanding natural diversity remains essential for uncovering how plants evolve detection of new pathogens and for successful deployment of disease resistance in crops (Barragan and Weigel 2021).

Plant immune receptors come in 2 main forms based on their domain architectures and cellular localization: surface-localized pattern recognition receptors (PRRs) and intracellular nucleotide-binding, leucine-rich repeat receptors (NLRs) (Jones and Dangl 2006; Ngou et al. 2022a). PRRs include receptor-like kinases (RLKs) and receptor-like proteins (RLPs) that bind to host-derived molecules, microbe-derived molecules, or pathogen-derived effector proteins present outside of the plant cell (Shiu and Bleecker 2003). Both RLKs and RLPs recognize diverse ligands with a variety of ectodomains, including leucine-rich repeats (LRRs), lysin motifs, malectin-like domains, and epidermal growth factor-like domains (Boutrot and Zipfel 2017; Albert et al. 2020). Upon ligand binding, a protein complex is formed between primary receptor, ligand, and RLK co-receptor(s), leading to kinase activation and downstream immune activation (Li et al. 2024b).

NLRs are functionally conserved across plants, animals, fungi, and bacteria in that they are able to perceive pathogen-derived effector proteins and induce immune signaling (Jones et al. 2016; Wojciechowski et al. 2022; Kibby et al. 2023). Plant NLRs are defined by their regulatory central NB-ARC (nucleotide-binding adaptor shared by Apaf-1, R proteins, and Ced-4) domain and series of C-terminal LRRs mostly implicated in ligand binding (Baggs et al. 2017). N-terminal signaling domains include coiled-coils (CC), Resistance to Powdery Mildew 8 (RPW8) domains, or Toll-Interleukin receptor (TIR) domains. Upon activation, NLRs form oligomeric structures bringing their signaling domains into activation (Jones et al. 2016; Lolle et al. 2020). Both CC-NLRs and RPW8-NLRs assemble into ion channel pores (Bi et al. 2021; Jacob et al. 2021), while tetrameric TIR-NLRs enable NADase enzymatic activities (Huang et al. 2022; Jia et al. 2022; Yu et al. 2022).

Functionally, NLRs and PRRs could be classified as sensors and helpers. Sensor NLRs can either directly bind pathogen effector proteins translocated into the host, guard host proteins targeted by effectors, or "bait" for the effectors with integrated domains (IDs) (Baggs et al. 2017; Bailey et al. 2018). Helper NLRs aid the activation of immune responses for a subset of sensor NLRs that belong to their regulatory networks or for their specific pairs (Adachi et al. 2019). Similarly, some PRRs act as sensors by binding to a particular epitope or ligand, whereas co-receptors from the SERK (somatic embryogenesis receptor kinase) family as well as SOBIR1 act as helpers, activating subsequent immune signaling (Snoeck et al. 2023). Immune signaling after PRR and NLR

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activation includes mitogen activated protein kinase signaling, Ca^{2+} burst, production of reactive oxygen species (ROS), transcriptional reprogramming, and, in case of most NLRs, programmed cell death called hypersensitive response (HR), allowing propagation of responses (Lolle et al. 2020; Jacob et al. 2023; Wang et al. 2023). Pathways activated by PRRs and NLRs work synergistically, with PRRs typically having a broader pathogen recognition range while NLRs have a more narrow, strain-specific but faster evolving response (Tian et al. 2021; Yuan et al. 2021; Ngou et al. 2022a).

Identifying functional PRRs and NLRs, defining their recognition range, and quantifying their diversity across evolutionary scales have led to multiple strategies of rational crop improvement (Jones et al. 2024). In this review, we focus on the "central dogma" as it relates to plant immune receptors and their associated diversity at the DNA, RNA, and protein level. We describe how available data have expanded from single genes to pangenomes and from individual gene expression studies to multi-tissue RNA-seq datasets. We propose that extreme combinatorial amino acid diversity in innate immune receptor repertoires on the plant pan-genome level fits the model of "anticipatory immunity," in which diversity is rapidly generated on a population level in anticipation of new pathogen challenge, leading to new recognition specificities and selection of functional alleles across generations. Accumulation of expression datasets together with advances in synthetic biology allow for new possibilities in engineering inducible disease resistance. Evolutionary, experimental, and computational structural analyses have facilitated the creation of designer receptors with resurrected, broader binding capabilities, or fully synthetic recognition specificities. With accumulation of training data, the future of incorporating generative machine learning could greatly expedite engineering efforts. Modern technologies have fueled basic research in *Arabidopsis thaliana* (hereafter Arabidopsis) for over 30 years and now have accelerated translation from model species into diverse crops, providing practical solutions for sustainable agriculture.

Episode 1. Hope for tomorrow: DNA diversity and anticipatory immunity

(By Chandler A. Sutherland and Ksenia V. Krasileva)

"A long time ago, in 119 million nucleotides far, far away…" we start our journey with DNA sequences. Most mutations are deleterious (Eyre-Walker and Keightley 2007; Chen et al. 2022), but immune systems present an evolutionary edge case in which high mutation is beneficial and even required to keep pace with rapidly evolving pathogens (Martincorena and Luscombe 2013; Müller et al. 2018). Across the tree of life, this has led to several radical evolutionary innovations, including emergence of molecular mechanisms of self-mutation and clonal selection on antibodies in vertebrates and CRISPR/Cas-based incorporation of extragenomic DNA in bacteria (Fig. 1) (Müller et al. 2018). The plant immune system is no exception in the need for rapid evolution. The stunning genetic diversity of plant immune receptors has long been appreciated and inspired multiple hypotheses and questions about its generation and maintenance.

Mystery is irresistible: early clues toward immune receptor diversity generation

The molecular study of NLRs and PRRs began with single gene cloning and diversity analysis in Arabidopsis (Bent et al. 1994; Grant et al. 1995; Gómez-Gómez and Boller 2000; Zipfel et al. 2006), tobacco (Whitham et al. 1994), and crops (Martin et al. 1993; Jones et al. 1994; Song et al. 1995; Meyers et al. 1998; Dodds et al. 2001). These studies established systems of immune receptors and their pathogen complements and provided molecular evidence for the gene-for-gene theory, where each functional plant immune gene is paired with a corresponding gene in the pathogen (Flor 1971). In this framework, the presence and activity of both gene partners is required for immunity. Even in this early work, the importance of population-level polymorphism in immune receptors to mediate coevolution of plants and their pathogens was recognized (Mode 1958; Flor 1971; Dodds 2023).

Genetic maps based on molecular markers revealed that many NLRs and PRRs in Arabidopsis (Kunkel 1996; Botella et al. 1997) and crops (Farrara et al. 1987; Islam and Shepherd 1991; Jones et al. 1993; Kesseli et al. 1994) are organized in genomic clusters. This clustered organization was hypothesized to increase the likelihood of tandem duplication, unequal crossing over, and gene conversion, driving structural and copy number variations (Parker et al. 1997; Parniske et al. 1997; Dixon et al. 1998; Michelmore and Meyers 1998; Thomas et al. 1998; Fritz-Laylin et al. 2005). However, clustered status is not a requirement for diversity, with singleton Arabidopsis NLR RPP13 maintaining extremely high amino acid diversity (Bittner-Eddy et al. 2000; Rose et al. 2004). Generally high amino acid diversity of the LRR domain of both NLRs and PRRs was highlighted and hypothesized to be maintained by diversifying and/or balancing selection (Hamilton et al. 1990; Parniske et al. 1997; Michelmore and Meyers 1998).

The first plant genome sequenced was Arabidopsis, allowing for genome-wide quantification of NLR and PRR copy numbers, types, and organization (The Arabidopsis Genome Initiative 2000; Dangl and Jones 2001; Shiu and Bleecker 2001). Characterization of NLRs and PRRs within and between species revealed high nucleotide diversity in the LRR region primarily driven by nonsynonymous mutations (Nordborg et al. 2005; Bakker et al. 2006; Li et al. 2024b). The NLR gene family in Arabidopsis includes the most polymorphic loci and contains the highest frequency of major effect mutations across the genome (Clark et al. 2007; Gan et al. 2011). For many immune-associated PRRs, diversity analysis has lagged behind genome generation due in part to the lack of known targets, making it difficult to functionally group receptors across species (Shiu and Bleecker 2001; Li et al. 2024b). However, mutational frequency is higher in PRRs associated with immunity than those involved in development (Ngou et al. 2024). It was proposed that the high copy number and clustered organization of immune receptors allowed for maintenance of functional recognition despite accumulation of the observed high polymorphism (Fig. 1) (Dangl and Jones 2001).

Non-model and crop reference genomes have become increasingly available with over 4,000 reference plant genomes currently deposited, allowing for interspecies quantification and comparison of PRR and NLRs across plants (Gao et al. 2018; Baggs et al. 2020; Man et al. 2020; Ngou et al. 2022b, 2024). The interspecies perspective on the evolution of immune receptors has shown lineage-specific expansions and contractions potentially mediated by domestication, pathogen pressure, and genome organization (Baggs et al. 2020; Giolai and Laine 2024; Gladieux et al. 2024). The expansion of genomic resources will facilitate identification of receptors with novel functional domains, likely leading to the innovation of new approaches for plant disease resistance.

Change is the essential process of all existence: pangenomic dimensions of diversity

The clustered organization, high incidence of structural variation, and polymorphic nature of immune receptors make them challenging to anchor to reference genomes (Fig. 1) (Jiao and Schneeberger 2020; Barragan and Weigel 2021). To overcome this, Resistance

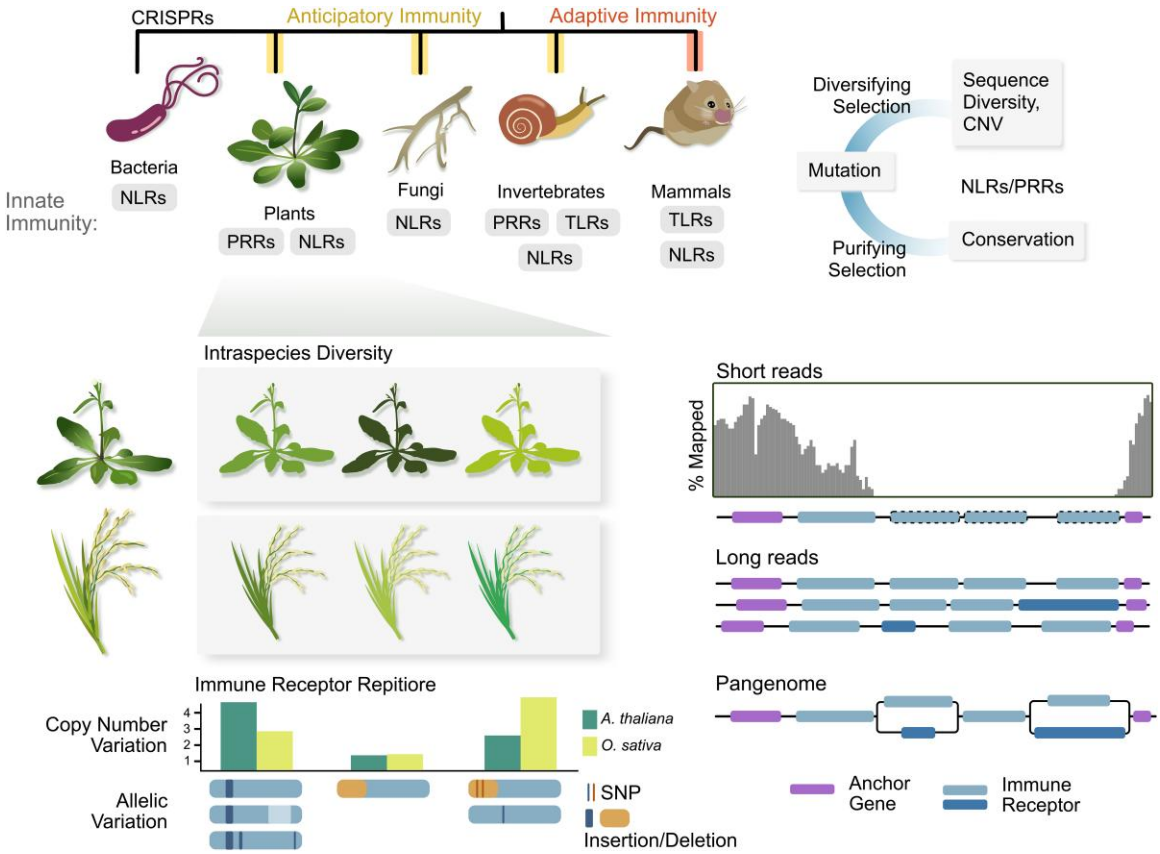

Figure 1. Immune receptors generate and maintain high diversity to manage diverse pathogenic threats. Across the tree of life, the pressure for diversity in immune receptors has resulted in multiple mechanisms for its generation including CRISPR/Cas-based systems in bacteria, adaptive immunity in vertebrates, and anticipatory immunity in invertebrates, plants, and fungi. Innate immune receptors include NLRs, PRRs, and toll-like receptors (TLRs). Within or across species, receptor number (copy number variation [CNV]) and allelic variation (single nucleotide polymorphisms and insertions and deletions) can be measured using genomic sequencing. Long-read sequencing and de novo reference assembly enables capture of diverse immune receptor content that was previously missed in reference-based genome assemblies using short-read sequencing. Anchor genes act as conserved, syntenic loci or blocks across related genomes that aid to compare variable gene content.

Gene Enrichment Sequencing (RenSeq) uses a combination of hybrid probe-based enrichment of NLR loci and long read sequencing to allow for examination of NLR diversity across and within species (Jupe et al. 2013). Application of RenSeq across 64 *Arabidopsis* accessions enabled a comprehensive, intraspecies view of NLR diversity (Van de Weyer et al. 2019). The portability of this technique has quickly translated to crop species and their wild relatives, enabling rapid cloning of resistance genes (Witek et al. 2016; Arora et al. 2019; Vendelbo et al. 2022), investigation of selective pressures related to domestication (Seong et al. 2020; Gladieux et al. 2024), and study of the relationship between ecological pressures and NLR gene content (Keepers et al. 2024).

If RenSeq unlocked the door to comprehensive immune receptor diversity characterization, recent long-read, de novo assembled pan-genomes have thrown it wide open. Long read pan-genomes of Arabidopsis now cover 167 distinct accessions (Jiao and Schneeberger 2020; Kang et al. 2023; Wlodzimierz et al. 2023; Lian et al. 2024; Teasdale et al. 2024), and pan-genome scale long-read assemblies are now available for many crops, including maize (Hufford et al. 2021) and rice (Shang et al. 2022), and their availability has recently been reviewed (Pucker et al. 2022; Shi et al. 2023). Long-read genomes provide the opportunity to investigate intraspecies PRR evolution, an opportunity unavailable with RenSeq data. Researchers are no longer limited in the availability and accuracy of sequences of plant immune receptors but in the ability to meaningfully analyze and draw conclusions from them in the pangenomic dimension (Barragan and Weigel 2021).

Phylogenetic grouping of pangenome sequences of PRRs and NLRs has shown high diversity at the solvent-exposed residues of the LRR domains, confirming single gene studies on a genomic level (Man et al. 2020; Prigozhin and Krasileva 2021; Trinh et al. 2023; Li et al. 2024b). Quantification of amino acid diversity by Shannon entropy in the model species of Arabidopsis, Brachypodium (Prigozhin and Krasileva 2021), and maize (Prigozhin et al. 2025) reveal in each a subset of NLRs evolving quite rapidly. Pangenomic, gene-family level analysis of NLRs has shown evidence of balancing and directional selection in rice landraces (Gladieux et al. 2024) and positive, diversifying selection in Arabidopsis driving NLR diversity (Van de Weyer et al. 2019; Sutherland et al. 2024). In Arabidopsis, this high diversity is associated with distinct genomic features and a predicted higher likelihood of mutation (Sutherland et al. 2024). Use of graph-based methods to define the genomic regions surrounding NLRs in 17 Arabidopsis accessions allowed for a holistic view of NLR evolution, reaffirming previous emphasis on local copy number variation, association of variable NLRs with transposable elements (TEs), and high polymorphism (Teasdale et al. 2024). Application of these tools developed in Arabidopsis to other crop species will reveal the extent to which these observations are conserved.

While mechanistic questions remain, long-read, pangenome approaches have supported and unified many early hypotheses on the maintenance and generation of immune receptor diversity.

In Arabidopsis, a "diversity in diversification" explanation helps to combine the contributions of large structural variations, fusions, deletions, gene conversion, point mutation, and TE insertions (Teasdale et al. 2024). Understanding the mechanisms of diversity generation, the sequence space of receptors, and the associated genomic contexts will allow us to boost the efficiency and success rate of receptor engineering, inspired by plants' natural genome engineering efforts.

Your focus determines your reality: exploiting de novo mutation events for the study of immune receptor evolution

Plant immune receptor diversity creates population-level resistance against pathogens, with variation in recognition between receptors preventing ubiquitous colonization by single pathogen strains (Hamilton et al. 1990; Dangl and Jones 2001; Bakker et al. 2006; Teasdale et al. 2024). We propose that plant immune systems, placed in the context of innate immune system evolution across the tree of life, follow a pattern of anticipatory immunity (Müller et al. 2018), where immune receptors are both more likely to mutate and are selected for high diversity. Mutation here is used in the broadest sense, encompassing structural variation and sequence polymorphism, reflecting all possible outcomes of DNA damage and repair. This immune system is anticipatory as opposed to adaptive because it includes higher mutational likelihood at immune receptors in anticipation of pathogen attack with classical Darwinian, population-scale selection as opposed to clonal, individual-scale selection.

High diversity generation specific to innate immune genes in anticipation of pathogens has been described in several invertebrates (Ghosh et al. 2011; Müller et al. 2018; Buckley and Dooley 2022) but has not been explicitly extended to plants. While the receptors are distinct in function and protein domain composition, the mechanisms of diversity generation and selective pressures are similar. For example, the SpTransformer (*SpTrf*) immune effector genes in sea urchins show extensive structural and single nucleotide sequence diversity (Buckley and Smith 2007; Oren et al. 2016a, 2019) and are often in clustered, repetitive gene families that have been compared with NLR clusters (Oren et al. 2016b). These repeat rich regions create genomic instability that promotes unequal crossovers, gene conversion, duplications and deletions, and mispairings that drive *SpTrf* diversity (Oren et al. 2016a; Barela Hudgell et al. 2024). There is evidence of both somatic recombination and mutation of the *SpTrf* family (Buckley et al. 2008; Oren et al. 2019) independent of exposure to immune elicitors, consistent with continuous diversity generation in anticipation of pathogen pressure. Though questions around targeting and mechanism remain specifically regarding somatic single nucleotide polymorphisms, we propose that the described "diversity in diversification" in plants fits into an anticipatory innate immunity model where diversity is generated through elevated mutation in selected immune receptors on a population level in anticipation of emergence of new pathogen strains.

An opportunity to test this hypothesis is highly accurate quantification of low frequency, de novo mutational events, as new mutations have minimal opportunity for selection to act. This allows for analyses of the underlying mutation distribution. Mutations are rare events on the population scale but occur constantly throughout plant development and growth. Previous applications of somatic mutation sequencing in Arabidopsis were used to compare mutations between tissues and characterize the mutational rate and spectrum of DNA repair knockouts (Wang et al. 2019c; Wu et al. 2020; Quiroz et al. 2024). However, improvements in ultra-accurate sequencing technology have decreased the variant calling error rate such that de novo mutations can be confidently quantified to the nanoscale (Abascal et al. 2021; Bae et al. 2023). Application of this technique to animal tissue has revealed several distinct signatures and rates of mutation associated with tissue types and age (Abascal et al. 2021; Moore et al. 2021; Cagan et al. 2022), but it has yet to be applied to plant nuclear DNA or broadly to innate immune gene evolution. Ultra-accurate mutation sequencing can be applied to both somatic and gametophytic tissues, with an opportunity to use pollen as an accessible representative of germline mutations. Structural variation can additionally be quantified de novo, posing the opportunity to observe the birth and death of immune receptors by copy number variation (Liu et al. 2024b). Arabidopsis is a natural starting point for adaptation of this technology to plants due to its small genome size and corresponding low cost per mutation, but future applications to crops will provide comparative insight. These improvements in sequencing technology will allow for direct quantification of mutation in immune receptors and help identify the mechanisms driving this diversity.

Episode 2. Temptation of response: expression variation and its regulation

(By Wei Wei and Chandler A. Sutherland)

Constitutively activated defense responses can cause severe cell damage and reduce plant growth, making precise regulation of immune receptor expression critical for balancing immunity and growth. While lineage-specific patterns in expression are observable even within species, common pathways for evolution of regulation can be surmised from studies across model plants and crops. Editing transcriptional regulation provides a promising opportunity for engineering broad-spectrum resistance.

Logic is the beginning of wisdom: immune receptor expression is induced by stimuli and can be tightly regulated

The first observed expression pattern for NLRs was induction in response to pathogens or other stress stimuli. In Arabidopsis, 74 out of 124 NLRs surveyed using microarray exhibited significant inductions by at least 1 pathogen, pathogen-related defense elicitors, or hormone treatment (Mohr et al. 2010). The 74 NLR genes varied in the types of pathogens or treatments they responded to, suggesting diverse regulatory pathways mediating NLR induction. Similarly, characterization of the NLR gene family in tomatoes revealed that 15 and 74 NLRs out of 321 were differentially expressed in early and late blight-infected leaves, respectively (Bashir et al. 2022). NLRs in monocot crop species such as maize and rice also exhibited expression changes induced by biotic and abiotic stresses (Ding et al. 2020; Hayford et al. 2024). When responding to pathogens, some NLRs showed differential expression between resistant and susceptible genotypes, pointing to the relevance of expression regulation to immunity (Bashir et al. 2022; Fick et al. 2022a). Pathogen perception triggered by specific NLRs can induce global NLR expression, suggesting a potential positive feedback loop to boost immunity (Mohr et al. 2010; Jacob et al. 2018). In both NLR-based hybrid incompatibilities (Bomblies et al. 2007; Atanasov et al. 2018; Barragan et al. 2021) and autoactive immune mutants (Yang et al. 2020, 2021), a large proportion of noncausal NLRs are induced.

Gene expression is in part regulated by the cis-regulatory elements located in promoters. A motif search of all Arabidopsis NLR promoters revealed enrichment of W-boxes, the binding site for WRKY transcription factors implicated in defense responses (Mohr et al. 2010). Mutagenesis of 3 W-boxes in the *RPP8* promoter greatly diminished its inducibility and resistance to pathogens, indicating their importance in regulating NLR expression (Mohr et al. 2010). NLR gene expression regulation has also been widely reported on the pre-transcription level (histone modification and DNA methylation) and post-transcription level (alternative splicing, premature transcription termination and small RNAs), which were recently reviewed (Lai and Eulgem 2018; Fick et al. 2022b).

Most immune associated PRRs appear to be developmentally and spatially regulated, particularly in the roots and shoots. In Arabidopsis, the flagellin receptor FLS2 and elongation factor Tu receptor EFR are expressed in the shoots as early as 3 weeks of age. In roots, only FLS2 was induced in the endodermis of the differentiated root, whereas EFR was not (Beck et al. 2014; Wyrsch et al. 2015; Emonet et al. 2021). In tomato, the flagellin receptor FLS3 and cold shock protein receptor CORE are expressed in foliar tissue as early as 4 and 6 weeks of age, respectively (Clarke et al. 2013; Wang et al. 2016). Conversely in the roots, FLS2, FLS3, and CORE displayed enhanced expression in seedlings during the early, but not late, differentiation state (Leuschen-Kohl et al. 2024). We hypothesize receptor sequences—and, more likely, expression variations—would explain the differential immune response across plant tissues or species previously observed (Clarke et al. 2013; Wei et al. 2018; Trinh et al. 2023).

The force is a balance between extremes: immune receptors exist across axes of regulation

With the increase of RNA-seq datasets across species and tissue types, the precise dynamics of immune receptor expression can be examined at scale. It was recently observed that high NLR expression in the absence of a pathogen is more common than previously recognized in Arabidopsis, tomato, barley, and wild wheat progenitor leaf tissues (Fig. 2) (Brabham et al. 2024b). High steady-state expression of rapidly evolving NLRs has been observed across tissues in Arabidopsis Col-0 (Sutherland et al. 2024), and several NLRs across the pangenome of maize show constitutive expression (Prigozhin et al. 2025). RLK expression in Arabidopsis and tomato has shown constitutive, tissue-specific, and developmentally regulated expression, whereas RLPs in Arabidopsis show constitutive and pathogen-inducible expression (Chuberre et al. 2018; Emonet et al. 2021; Steidele and Stam 2021). Therefore, while some immune receptors may be under tight transcriptional control to prevent autoimmunity or more general reductions in fitness, there are many NLRs and some PRRs that are expressed highly in the absence of pathogens, and this expression is in some cases required for their function as sensors of effectors (Bieri et al. 2004; Brabham et al. 2024b).

Restricting expression of NLRs to the tissues with the highest pathogen pressure would reduce fitness costs associated with high receptor expression (Contreras et al. 2023; Lüdke et al. 2023). Two examples in tomato support this hypothesis: the *I-2* gene provides immunity against vascular wilt and shows vascular tissue-specific expression (Mes et al. 2000), and the *NRC6* gene cluster that confers resistance to cyst and root-knot nematodes has root-exclusive expression (Lüdke et al. 2023). Spatial regulation of expression can also be driven by developmental tradeoffs: altering of *FLS2*-expressing cell types in Arabidopsis caused collapsed meristem cells and inhibited root growth (Emonet et al. 2021). At the gene family level, NLRs in Brassicaceae including Arabidopsis show shoot-skewed NLR expression, while tomato, maize, rice, and several legume species show root-skewed NLR expression (Munch et al. 2018; Lüdke et al. 2023).

Immune receptor expression level is shaped by downward selective pressure driven by fitness costs associated with excessive expression and upward selective pressure of the minimum dosage required for functionality (Fig. 2). Depending on the mode of action and likelihood of autoactivity, individual receptors have different upper and lower bounds of tolerable expression. The NLRs Sr33 and Sr50 are moderately autoactive on the protein level and constitutively high gene expression exacerbates the autoactivity (Tamborski et al. 2023). In this case, pathogen-specific induction of NLRs, in combination with low basal expression, would prevent autoimmunity while maintaining their functionality for defense activation upon pathogen attack. NLRs in sensor-helper pairs are in tight co-evolutionary relationships, as unbalanced expression of the 2 NLRs can spuriously activate the immune system. These NLR pairs are usually found to be co-localized in the genome, with head-to-head orientation, supposedly being co-regulated by the same promoter (Shimizu et al. 2022; Yang 2024). However, multiple transgene copies of the direct recognition NLR *Mla7* in barley are required for complementation of a susceptible background (Brabham et al. 2024b), indicating a requirement of high expression for functionality. Analysis of the pan-transcriptome of maize showed orthologous NLRs with high variation in expression: RppM and RppC-like clade members showed silent, tissue specific, or constitutive expression across lines (Prigozhin et al. 2025). Understanding the relationship between expression, mechanism, and functionality of NLRs will greatly assist engineering efforts and successful implementation in crops (Fig. 2).

Two theories have emerged for the relationship between expression and evolvability of immune receptors. For an immune receptor under diversifying selection to evolve new recognition specificity, it can either be lowly expressed while accumulating mutations and then be de-repressed to higher expression once a functional allele evolves (Shivaprasad et al. 2012; Zhang et al. 2016; Brabham et al. 2024b), or it can begin constitutively expressed and evolve regulation after functionality (Prigozhin and Krasileva 2021; Sutherland et al. 2024). Low expression of a rapidly evolving receptor would allow for a higher tolerance of mutations with slightly deleterious effects until functionality is achieved, as supported by the presence of microRNA families capable of silencing multiple NLRs simultaneously in pathogen-free tissues (Shivaprasad et al. 2012; Zhang et al. 2016) and the enrichment of functional, presumably not deleterious NLRs in constitutive expression states (Brabham et al. 2024b). Alternatively, high expression levels observed across highly variable NLRs (Brabham et al. 2024b; Sutherland et al. 2024) could compensate imperfect recognition or low activity of actively evolving receptors and may increase evolvability through increased likelihood of mutation associated with transcription (Staunton et al. 2023) and strengthened effect of individual mutations on fitness, increasing the efficacy of selection (Bódi et al. 2017; Payne and Wagner 2019). However, it is likely the variation in expression drives evolvability of immune receptors (Capp 2021; Prigozhin et al. 2025), with both theories acting in parallel on different receptors depending on the initial expression state and the strength of selection (Fig. 2). High throughput analysis of the relationship between expression levels and fitness of immune receptors of multiple types will provide the evidence required to resolve this thorny evolutionary trajectory.

NLR abundances are also highly regulated at the post-translational level. NLR proteins can be tagged by ubiquitin

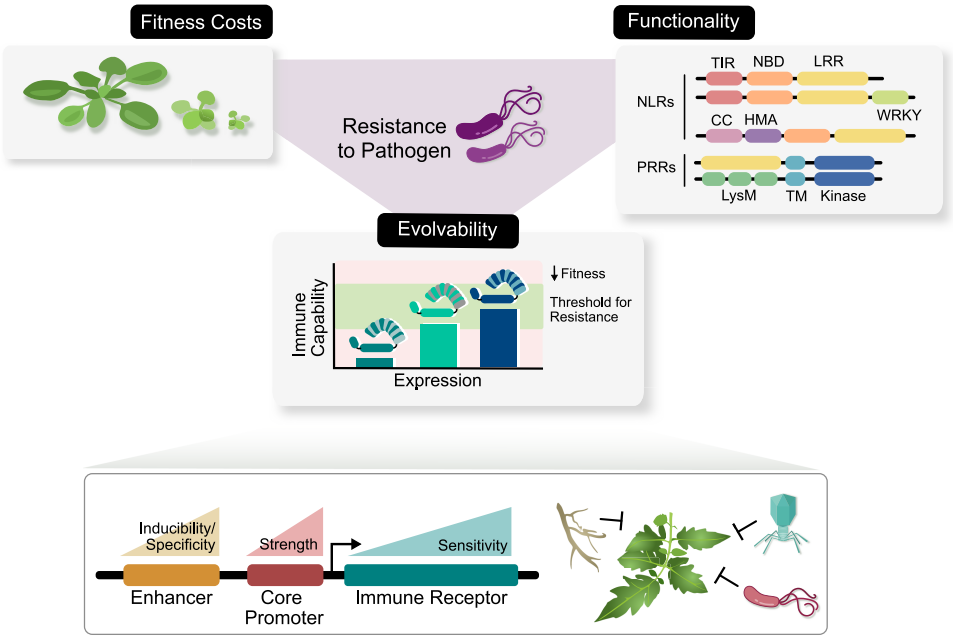

Figure 2. Different selective pressures on immune receptors can lead to diverse expression patterns and inform engineering efforts for resistance to pathogens. Each receptor has a minimal threshold of expression to effectively monitor for the pathogen and an upper threshold of expression associated with fitness costs. Different receptors have diverse modularity that can alter functionality and tolerable range of expression. Precise control of expression by fine-tuning cis-elements in enhancers and core promoters provides a platform for engineering broad-spectrum resistance against pathogenic threats. For functionality, domains are labeled as the following: CC, coiled-coil; NB, nucleotide-binding; LRR, leucine-rich repeat; HMA, heavy-metal-associated; TIR, Toll-Interleukin receptor; TM, transmembrane.

molecules, marking them for degradation by proteasome. The turnover of NLR proteins SNC1 (SUPPRESSOR of NPR1-1, CONSTITUTIVE 1) and RPS2 (RESISTANT TO P. SYRINGAE 2) are controlled by the E3 ubiquitin ligase complex SKP1-CULLIN1-F-box SCF^{CPR1} and loss of function of the *CPR1* led to higher accumulation of NLR proteins and autoimmune responses (Cheng et al. 2011; Huang et al. 2016). Several studies in Arabidopsis showed that molecular chaperones play essential roles in stabilizing NLR proteins. Chaperone complex formed by HSP90 (heat shock protein 90kD), SGT1 (Suppressor of G2 allele of skp1), and RAR1 (Required for Mla12 resistance) could associate with NLR proteins such as RPM1, N, and Rx, and were required for proper steady-state NLR levels (Kadota et al. 2010). These chaperones and co-chaperones are conserved across eukaryotic organisms and might serve as a conserved role for maintaining NLR homeostasis in both plants and mammals (Kadota et al. 2010). Although components regulating NLR protein abundance mentioned above have been identified and their functions elucidated, many remain unknown. Moreover, it is still unclear how universally applicable these regulatory mechanisms are across different NLR proteins and organisms.

I am one with the force, and the force is with me: opportunities and challenges in immune receptor expression engineering

Recent advancements in understanding the evolution of spatial and temporal expression can assist in the identification and engineering of functional immune receptors. For identification, constitutive expression can be used as a selection criteria to enrich for functional NLRs genome-wide or in map-based NLR cloning (Kawashima et al. 2016; Brabham et al. 2024b), and tissue specificity can be related to pathogen specificity, narrowing the search space for resistance (Contreras et al. 2023; Lüdke et al. 2023). For example, shoot-specific expression of an engineered defense-related gene in rice largely mitigated the fitness cost of constitutive defense, as it concentrated the immune responses to tissues threatened by their target pathogens (Molla et al. 2016). The development of spatial and single cell RNA-seq will provide high-resolution expression atlases (Zhu et al. 2023) and will help identify the underlying regulatory DNA sequences, bringing immune receptor engineering to unprecedented levels of precision. While the idea of engineering inducible expression of immunity has been explored for over 2 decades, it faced challenges in deployment as most approaches required generation of transgenic plants and many of the first promoters identified had fitness effects (Honee et al. 1998; Stuiver and Custers 2001; Li et al. 2020). With the expansion of expression datasets and advancements in genome editing and synthetic biology, there is renewed interest in deploying inducible expression for engineering broad-spectrum resistance (Wei et al. 2024).

The specificity of interaction between NLRs and effectors makes it challenging to engineer receptors for broad spectrum resistance. As an alternative strategy to protein engineering, several studies have explored control of transcription. For example, autoactive NLRs or elicitors of NLRs can be deployed in plants under pathogen-inducible (PI) promoters (Fig. 2). This strategy has been successful against pathogens harboring transcription activator-like (TAL) effectors, such as *Xanthomonas* and *Ralstonia* (Zeng et al. 2015; Gallas et al. 2024). Stacking effector binding elements in the promoter drives the expression of a cell death-inducing NLR gene *Xa10* in rice, enabling induction of resistance by multiple different *Xanthomonas oryzae* strains (Zeng et al. 2015). However, it is challenging to apply these synthetic cassettes against other pathogens because the transcription induction depends on TAL effectors. The tobacco promoter *hsr203j* shows conserved induction by pathogens of several classes and was harnessed to regulate an HR-inducing elicitor in tobacco, producing broad-spectrum resistance (Keller et al. 1999). Recently, an

autoactive mutant of *Sr33* and the intrinsically autoactive *Sr50* were fused with PI promoters in tomato for conferring broad-spectrum resistance (Wei et al. 2024). Critical to the success of this effort was pairing autoactive NLRs of varying sensitivities with PI promoters optimized for strength and inducibility to balance immunity and fitness costs (Fig. 2).

Using PI promoters to drive HR-inducing components is an actionable strategy for conferring broad-spectrum resistance but remains challenging. Widely adopting a transcriptional engineering strategy in crops requires precise transcriptional regulation specifically responsive to pathogens and not other stressors, particularly abiotic stress. Unintended induction of HR in response to other stimuli than pathogens will impose tremendous fitness costs on crops. Some PI promoters are also developmentally regulated, leading to unintended induction of NLR expression and autoimmunity at specific developmental stages (Honee et al. 1998; Wei et al. 2024). Comprehensive examination of transcriptomic resources across different tissues, developmental stages, and stress responses is necessary to mine for promoters specific to pathogen response. In addition, the advancement of synthetic biology tools has empowered engineering of transcriptional regulation. For example, promoter engineering at different scales could fine tune the expression precisely to balance leaky expression and induced immunity (Jores et al. 2021; Wei et al. 2024). Designing transcription circuits that display both pathogen inducibility and tissue specificity would further reduce the fitness cost of defense (Brophy et al. 2022). Several successful cases of engineering induced resistance also suggest adding post-transcriptional or translational regulations in addition to inducible promoters might be required to minimize undesired expression (Gonzalez et al. 2015; Xu et al. 2017). Manipulation of spatial, temporal, and inducibility of immune receptor expression, aided by characterized promoter libraries, represents an exciting opportunity for the future of plant immune engineering.

Episode 3. The rise of ultimate power: immune receptor engineering

(By Kyungyong Seong and Danielle M. Stevens)

Both genetic and regulatory changes are reflected in protein structure and function. In an endless arms race, pathogens evolve effectors to disarm plants, often adapting more rapidly and divergently than plants can respond. In monocultural fields, the breakdown of resistance can destroy entire populations. The ultimate power to overcome threats to food security rises through precise introduction of desired changes in immune receptors and rapid deployment of diverse germplasm.

A little more knowledge lights our way: paving the path to receptor engineering

Since the discovery of the first immune receptors, a central goal of our community has been to engineer receptors for improved, altered, or broadened ligand specificity. Early engineering endeavors, often using Arabidopsis and other model organisms, relied primarily on classical techniques including random mutagenesis, chimeras, and domain swaps leading to altered ligand perception (Wulff et al. 2001; Mueller et al. 2012; Steinbrenner et al. 2015; Helft et al. 2016), enhanced immune responses (Harris et al. 2013; Sueldo et al. 2015), and gain of effector recognition (Segretin et al. 2014; Huang et al. 2021). Allelic variation studies further clarified receptor function, residues required for ligand binding, and protein complex formation and stability (Dunning et al. 2007). The overarching aim remains to amplify intended immune outcomes while minimizing unintended consequences (Fig. 3). Achieving this requires precise and effective engineering strategies, together with continued accumulation of knowledge in protein stability during complex formation for PRRs (Dunning et al. 2007; Li et al. 2024a) and addressing challenges posed by intricate interactions of NLRs.

The debut of AlphaFold 1 in 2018 marked an important leap in protein structure prediction, demonstrating the transformative potential of deep learning in biology (AlQuraishi 2019). With the release of AlphaFold 2 in 2021, our field witnessed a paradigm shift in our understanding of immune receptors, effectors, ligands, and their interactions (Evans et al. 2021; Jumper et al. 2021; Abramson et al. 2024; Li et al. 2024b). Recent studies used AlphaFold 2 to explore the structural diversity of N-terminal domains of NLRs within *Plantae* (Chia et al. 2024) and LRR domains in maize (Prigozhin et al. 2025). AlphaFold 3 further enabled the construction of putative CC-NLR resistosome structures (Madhuprakash et al. 2024). In parallel, AlphaFold has expanded effector biology to the structural universe, notably improving insights in effector diversity, regulation, evolution, and interaction, previously difficult to decipher from primary sequences alone (Seong and Krasileva 2021, 2023; Rocafort et al. 2022; Derbyshire and Raffaele 2023; Homma et al. 2023; Yan et al. 2023; Asghar et al. 2024; Mukhopadhyay et al. 2024; Yu et al. 2024a). With its growing integration to receptor engineering, AlphaFold has begun to reshape our approach to develop precise and effective engineering strategies.

We are what they grew beyond: pushing the boundaries of precision LRR engineering

The structural determination of immune receptors in Arabidopsis has shaped our understanding of receptor function and engineering potential. The first structurally resolved RLKs include the flagellin receptor FLS2 and chitin receptor CERK1, revealing critical ligand-binding residues and receptor-ligand interactions (Table 1) (Liu et al. 2012; Sun et al. 2013). Nearly a decade later, the resistosome structures of NLRs ZAR1 and RPP1 in Arabidopsis demonstrated the mechanisms underlying NLR activation and effector recognition by the LRR domain (Table 1) (Wang et al. 2019a, 2019b; Ma et al. 2020). Only recently, structural determinations of NLRs from crop species have expanded these insights, enabling rational engineering of immune receptors (Table 1).

Availability of receptor complex structures has been instrumental to narrow the enormous mutational landscapes for precise engineering. Consider the NLR Sr50 as an example (Mago et al. 2015): it directly recognizes its effector through its LRR domain, which contains approximately 450 residues. Rational engineering begins with identifying key effector-binding residues within this extensive sequence space. After selecting 12 interacting residues (Tamborski et al. 2023), modifications could still allow up to 20^{12} allelic variants, comparable to antibody diversity (Briney et al. 2019). Structural insights significantly contribute to addressing these challenges by identifying key residues and informing rational introduction of mutations. For instance, the structure of Arabidopsis FLS2 bound to its ligand flg22 revealed the ligand-binding interface and residues within the LRR domain, enabling modifications that improved flg22 binding affinity and gain of perception of previously evaded variants (Sun et al. 2013; Fürst et al. 2020; Wei et al. 2020; Li et al. 2024a; Zhang et al. 2024a). Similarly, the solved structures of NLRs, such as Sr35

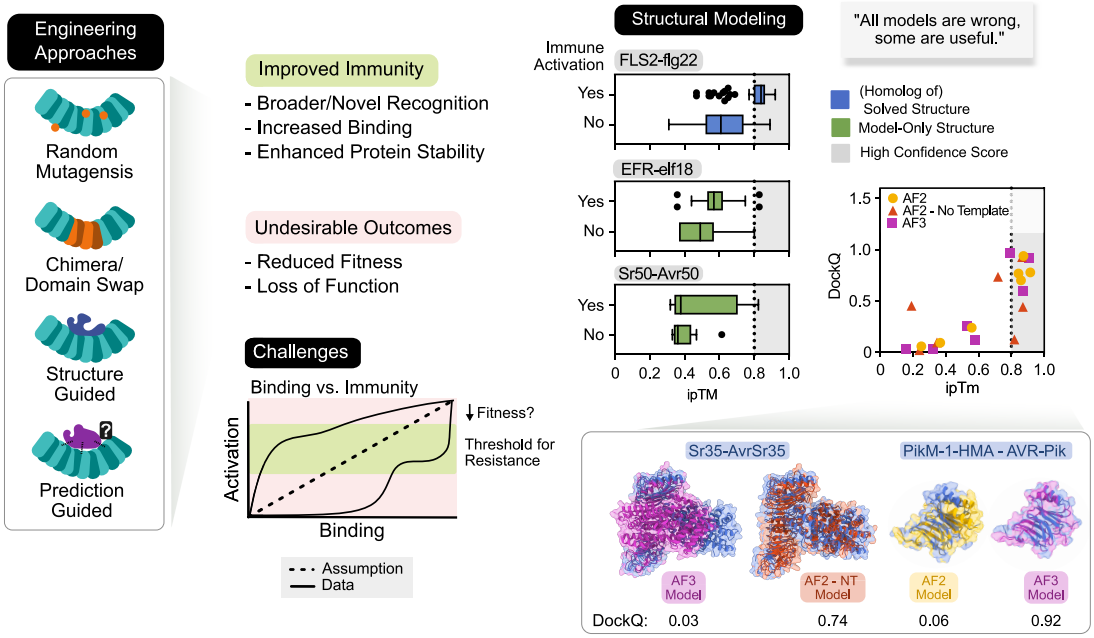

Figure 3. Combination of techniques including structural modeling enable engineering of immune receptors. Engineering approaches aim to improve immune responses but could lead to undesirable outcomes. In particular, our understanding of the relationship between ligand binding and immune activation is yet incomplete, making it challenging to establish strategies to overcome the unintended consequences. Structural prediction enables hypothesis generation of receptor-ligand interactions and may guide engineering endeavors. The interface predicted template modeling (ipTM) score above 0.8 often indicates reliable AlphaFold prediction and correlates with immune responses for the FLS2-flg22 interactions in Arabidopsis. However, this correlation is not strong in other assessed receptor-ligand interactions. The performance of AlphaFold 2 and 3 vary significantly in predicting NLR/ID-effector pairs (AF2, AlphaFold 2; AF2-NT, AlphaFold 2-No effector Template; AF3, AlphaFold 3). When AlphaFold models are compared with experimental structures, their accuracy, measured by DockQ scores (0: inaccurate, and 1: accurate), shows that selection based on high ipTM scores often indicate reliability but does not always guarantee reliable models. Predicted structures are good hypotheses but should be interpreted with caution.

from einkorn wheat and MLA13 from barley (Table 1), in complex with their effectors allowed precise inference of effector-binding residues and facilitated direct transfer or expansion of recognition specificity to other homologs (Förderer et al. 2022; Zhao et al. 2022; Lawson et al. 2025). These examples demonstrate how experimentally determined PRR-ligand and NLR-effector complex structures are driving precision engineering.

Recent advances in computational modeling, particularly AlphaFold, are further advancing receptor engineering. Two independent studies used AlphaFold models to assist the selection and design of altered ligand recognition in FLS2, where a small number of substitutions resulted in gain of recognition of flg22 epitopes (Li et al. 2024a; Zhang et al. 2024a). For NLRs, AlphaFold accurately predicted the effector binding site of MLA3 in barley, subsequently enabling its precise transfer to Sr50 (Gómez De La Cruz et al. 2024). Similarly, AlphaFold reliably predicted the complex structures of Sr50 variants bound to their effector AvrSr50, aiding in the design of novel recognition for the escape effector mutant AvrSr50^{QCMJC} (Ortiz et al. 2022; Seong et al. 2025). These examples suggest that structure prediction has the potential to accelerate LRR engineering, particularly for receptor-ligand pairs lacking experimentally determined structures.

Despite technical advancements in experimental and predictive structural determination, not all receptor-ligand structures can be accurately characterized. An alternative approach is to leverage the evolutionary information that plants, as bioengineers, have naturally accumulated. Quantifying intraspecies receptor sequence diversity, as demonstrated for Arabidopsis, can aid in inferring residues rapidly changing for ligand binding (Van de Weyer et al. 2019; Prigozhin and Krasileva 2021). In wheat, this framework aided in the transfer of recognition specificity of NLR Sr50 to closely related Sr33 (Tamborski et al. 2023), highlighting its applicability in receptor engineering in crops. Similarly, phylogenetic comparisons and ancestral reconstruction of the bean receptor INR prioritized residues that could alter perception of the caterpillar-associated molecule inceptin (Snoeck et al. 2022). In practice, sequence- and structure-based approaches are not independent but complementary. The effective integration of computational modeling, evolutionary insights, and high-throughput screening will continue to push the boundaries of LRR engineering in the near future.

Things are only impossible until they're not: expanding ingenuity of decoy engineering approaches

The sequencing and annotation of the Arabidopsis genome facilitated early studies and analyses of Arabidopsis NLRs, revealing atypical fusion of domains into NLRs, such as RRS1 (The Arabidopsis Genome Initiative 2000; Deslandes et al. 2002, 2003; Meyers et al. 2003). In parallel, accumulated studies in Arabidopsis NLRs RPS2, RPM1, RPS5 and ZAR1 supported indirect NLR recognition mechanism in which NLRs monitor modifications of host proteins by effectors instead of directly recognizing them (Axtell and Staskawicz 2003; Mackey et al. 2003; Shao et al. 2003; Wang et al. 2015). These early observations became foundations to guardee, decoy, and ID models (Jones and Dangl 2006; van der Hoorn and Kamoun 2008; Cesari et al. 2014), in which pathogen target proteins are re-purposed as effector baits to effectively trigger immune responses, as either individual proteins or integrated into NLRs.

Some of the most advanced engineering of NLR systems with guardees and decoys began with well-characterized Arabidopsis

Table 1. Representative structures of NLRs and PRRs

PDB Accession[a]	NLR/ PRR	Type	Organism	Effector/ Ligand	Organism	References
4EBZ	CERK1	LysM-RLK	*Arabidopsis thaliana*	chitin		(Liu et al. 2012)
4MN8	FLS2	LRR-RLK	*Arabidopsis thaliana*	flg22		(Sun et al. 2013)
6J5T	ZAR1	CC-NLR	*Arabidopsis thaliana*			(Wang et al. 2019a, 2019b)
7CRC	RPP1	TIR-NLR	*Arabidopsis thaliana*	ATR1	*Hyaloperonospora arabidopsidis*	(Ma et al. 2020)
7JLU	Roq1	TIR-NLR	*Nicotiana benthamiana*	XopQ	*Xanthomonas euvesicatoria*	(Martin et al. 2020)
7DRC	RXEG1	LRR-RLP	*Nicotiana benthamiana*	XEG1		(Sun et al. 2022)
7XE0	Sr35	CC-NLR	*Triticum monococcum*	AvrSr35	*Puccinia graminis* f. sp. *tritici*	(Zhao et al. 2022)
7XC2	Sr35	CC-NLR	*Triticum monococcum*	AvrSr35	*Puccinia graminis* f. sp. *tritici*	(Förderer et al. 2022)
9FYC	MLA13	CC-NLR	*Hordeum vulgare*	AVR_{A13}-1	*Blumeria hordei*	(Lawson et al. 2025)
8RFH	NRC2	CC-NLR	*Nicotiana benthamiana*			(Selvaraj et al. 2024)
9FP6	NRC2	CC-NLR	*Nicotiana benthamiana*			(Madhuprakash et al. 2024)
8XUV	NRC2	CC-NLR	*Solanum lycopersicum*			(Ma et al. 2024)
9CC9	NRC4	CC-NLR	*Nicotiana benthamiana*			(Liu et al. 2024a)
8ZF0	ADR1	RPW8-NLR	*Oryza sativa*			(Wu et al. 2024)
8ZW9	ADR1	RPW8-NLR	*Arabidopsis thaliana*			(Yu et al. 2024b)
8YN0	NRG1	RPW8-NLR	*Arabidopsis thaliana*			(Huang et al. 2025)
8YL7	NRG1	RPW8-NLR	*Arabidopsis thaliana*			(Xiao et al. 2025)

[a]Only 1 structure is associated with each study in this table. Additional structures can be identified within each entry in the Protein Data Bank (PDB).

NLR and receptor-like cytoplasmic kinase systems, specifically RPS5/PBS1-like (DeYoung et al. 2012) and ZAR1/ZED1-like (Lewis et al. 2013). Upon cleavage by bacterial effector AvrPphB, PBS1 undergoes a conformational change that is recognized by RPS5 leading to the activation of immune responses (DeYoung et al. 2012). This activation mechanism has been deployed for engineering recognition of diverse pathogen proteases: substituting the proteolytic site of PBS1 with that of another effector AvrRpt2 from bacterial pathogen *Pseudomonas syringae* or the NIa viral protease of Tobacco etch virus led to partial or full resistance to pathogens (Kim et al. 2016). Most recently, it was shown that the partial resistance can be rescued by overexpressing engineered PBS1 and RPS5, and this approach has been successfully extended to soybean (Helm et al. 2019; Pottinger et al. 2020).

Similar to RPS5, Arabidopsis NLR ZAR1 monitors a diverse set of receptor-like cytoplasmic kinases like ZED1 targeted by the effector HopZ1a (Lewis et al. 2013; Seto et al. 2017). Recent natural diversity analyses across 1,001 Arabidopsis ecotypes revealed key residues governing ZAR1-ZED1 interactions (Baudin et al. 2021). Extending these analyses to other kinases revealed that ZAR1 can interact with the majority of ZED1-like kinases in Arabidopsis and key residues contributing to binding specificity (Diplock et al. 2023). In combination with mutational screens, natural diversity data from Arabidopsis was leveraged to allow tomato ZAR1 to recognize HopZ1a (Diplock et al. 2024). As ZAR1 is an ancient and highly conserved NLR across angiosperms (Gong et al. 2022), the engineering application can expand to other crops.

The majority of NLR-ID engineering efforts have focused on RRS1 in Arabidopsis (Narusaka et al. 2014; Le Roux et al. 2015; Sarris et al. 2015; Zhang et al. 2017; Mukhi et al. 2021) and RGA5 and Pik-1 in rice (Ashikawa et al. 2008; Cesari et al. 2013; Maqbool et al. 2015; Ortiz et al. 2017; Guo et al. 2018; Sugihara et al. 2023), but continued computational and experimental characterization of NLR-IDs in crops will allow for expansion of engineering efforts (Sarris et al. 2015; Kroj et al. 2016; Bailey et al. 2018; Marchal et al. 2018, 2022; Wang et al. 2020). Conceptually, NLR-ID engineering parallels LRR engineering, in that experimentally determined structures of ID-effector complexes, complemented by sequence analyses, have largely informed the selection and modification of effector binding residues (De la Concepcion et al. 2018, 2019; Bentham et al. 2021, 2023; Liu et al. 2021b; Cesari et al. 2022; Saado et al. 2024; Zhang et al. 2024b). Advances in structure-guided precision engineering and diverse tactics in NLR-IDs are well highlighted in recent reviews (Marchal et al. 2022; Cadiou et al. 2023; Zdrzałek et al. 2023).

Distinct from LRR engineering strategies, the homologous relationships between IDs and effector-targeted host proteins provides an effective nature-guided engineering approach. Identifying host targets and elucidating their interactions with effectors allows the transfer of specific effector-binding residues to NLR-IDs. For example, the crystal structure of the effector AVR-Pik bound to its rice host protein HIPP19 informed mutations expanding the recognition specificity of Pik-1 against stealthy effector variants (Maidment et al. 2021, 2023). Similarly, structural insights from the interaction between the effector Pwl2 and the rice protein HIPP43 enabled engineering the Pikm-1 receptor with broad pathogen recognition capabilities (Zdrzałek et al. 2024). Alternatively, IDs in NLRs can be replaced by their homologs targeted by effectors (Zhu et al. 2025). As such, identifying additional effector-targeted host proteins, such as OsHIPP20 (Oikawa et al. 2024), provide further avenues for engineering opportunities.

Modularity and compactness of IDs also facilitated scalable binder design strategies. Yeast cell surface display screening, a technique frequently used for mammalian nanobody evolution (McMahon et al. 2018), has been adapted for directly evolving higher-affinity IDs in vivo (Rim et al. 2024). In this technique, mutagenized IDs are displayed on the yeast cell surface and screened against targeted effectors, allowing selection of improved binders via fluorescence-activated cell sorting. Additionally, the pikobody approach expanded the plant immune engineering toolkit by leveraging the mammalian immune systems (Kourelis et al. 2023). In this method, nanobodies capable of binding to fluorescent proteins were integrated into Pik-1, replacing its original ID. The resulting pikobody (Pik-nanobody fusion) successfully induced immune responses in plants in the presence of fluorescent proteins, demonstrating that modular IDs capable of effector binding could be derived from the mammalian immune system. Ensuring the proper integration of engineered or designed domains without triggering autoimmunity and compromising immune responses remains a key challenge in NLR-ID engineering. Nevertheless, these strategies broaden

the toolkit available to engineers, enabling the development of novel synthetic solutions.

May the fold be with you: new structural quests for immune receptors and effectors

The rapid expansion of predicted structure databases, coupled with the advance in structural similarity search algorithms presents new opportunities to explore the structural universe (Varadi et al. 2022; van Kempen et al. 2024). Molecular mimicry has emerged as viable tactics in pathogen evolution, where pathogen effectors structurally mimic host proteins and hijack host signaling pathways (Gao et al. 2021; Fu et al. 2025). Likewise, certain NLRs' LRR domain mimics the structure of effector-targeted host proteins, effectively trapping effectors to trigger immune responses (Chen et al. 2023; Gómez De La Cruz et al. 2024). Examples include the pepper NLR Tsw, which possesses an extended LRR domain resembling hormone receptors (Chen et al. 2023), and the barely NLR MLA3 that mimics the host protein HIPP43 to bait its effector (Brabham et al. 2024a; Gómez De La Cruz et al. 2024; Zdrzałek et al. 2024). With millions of predicted structures now available, large-scale structural similarity comparisons can further illuminate this molecular arms race between plant immunity and pathogen effectors.

The direct applicability of AlphaFold for modeling and understanding LRR-ligand or LRR-effector interactions is an active area of exploration (Fig. 3). For PRRs, the analysis of FLS2-flg22 interactions indicated that a prediction confidence (ipTM) threshold of 0.8 could reliably distinguish the FLS2-flg22 variant pairs that could induce immune responses in Arabidopsis from the pairs that could not (Fig. 3) (Li et al. 2024a). However, when assessing recognition prediction for EFR-elf18 that do not have experimentally determined structure using the same threshold, accuracy was very low (Fig. 3) (Colaianni et al. 2021; Parys et al. 2021; Stevens et al. 2024; Trinh et al. 2024). Similarly, an ipTM threshold of 0.8 could differentiate reliable from unreliable AlphaFold predictions for the NLR Sr50, yet reliability of models did not always guarantee immune recognition in plants (Seong et al. 2025). Here, we present our own assessments comparing predicted LRR/ID-effector models against experimentally determined structures. Our analyses reinforced that high ipTM thresholds typically, but not always, indicate reliability, as reported previously (Fig. 3) (Supplementary Table S1) (Evans et al. 2021; Wee and Wei 2024). Therefore, while AlphaFold has enabled structural modeling attempts for many known NLR-effector pairs lacking experimental structures (Fick et al. 2024; Wang et al. 2024), these predictions require careful validation through additional experimental data and may not alone be considered as definitive evidence of immune recognition.

Our understanding of AlphaFold's predictive capabilities will continue to evolve through ongoing research. For instance, AlphaFold 2 failed to accurately predict the complex structure of the NLR Sr50 and its effector AvrSr50 but successfully modeled reliable structures for Sr50 variants bound to AvrSr50 (Gómez De La Cruz et al. 2024; Seong et al. 2025). Intriguingly, these receptor variants contained as little as a single amino acid substitution from the wild type, yet this seemingly small change dramatically improved prediction accuracy. This highlights a fascinating yet unexplored aspect of AlphaFold's sensitivity to minor mutations in protein complex structure prediction. Computational mutational screening may uncover underlying principles governing this predictive behavior, and their identification may enable the recovery of receptor-ligand structures that cannot be currently predicted with high accuracy.

Continued algorithmic advancements, such as AlphaFold 3, promise improvements in accurate structural modeling. However, despite the release of AlphaFold 3, AlphaFold 2 remains valuable, particularly when experimentally determined structures are needed as templates to improve prediction accuracy (Fig. 3) (Outram et al. 2024). Moreover, AlphaFold 2, along with the more accessible ColabFold (Mirdita et al. 2022), offers tunable parameters, such as number of random seeds, recycles, and ensembles, which can enhance the predictive power (Jumper et al. 2021; Agarwal and McShan 2024). Therefore, researchers can explore both options to identify the best model suited to the characteristics of their system and specific biological question.

Looking ahead, refining AlphaFold predictions for PRR-ligand and NLR-effector complexes will greatly benefit from broader community collaboration. By standardizing benchmarking protocols and experimentally validating predicted structures, our community can more effectively assess AlphaFold's performance and identify pathways for improvement. Expanding the set of reliably predicted receptor-ligand complexes can rapidly deepen our understanding of molecular interactions and enhance predictive capabilities in protein design, while experimental structures provide essential ground-truth over time. Embracing not only successes but also failures in this collective structural endeavor is essential to complete our structural quests. After all, all models are wrong, but some are useful.

To boldly go where no one has gone before: leveraging deep learning in plant immunity

Beyond protein structure prediction, machine learning—particularly deep learning—has rapidly expanded our toolkit for exploring protein biology. Geometric deep learning enables protein surface analyses to identify protein-protein interaction sites (Gainza et al. 2020; Tubiana et al. 2022; Krapp et al. 2023). Advances in generative algorithms like ProteinMPNN and RFDiffusion have made it possible to accurately design primary sequences and protein structures (Dauparas et al. 2022; Watson et al. 2023). Additionally, protein language models have shown remarkable potential with diverse applications, such as elucidating evolutionary principles underlying viral mutations and aiding antibody design by accurately predicting mutations that enhance affinity (Hie et al. 2021, 2022, 2024; Lin et al. 2023; Ruffolo and Madani 2024). Such applications can potentially extend to NLRs and effectors, as well as PRRs and ligands, where language model-driven predictions could link specific mutations to their corresponding roles, functions, and interactions. Notably, evolutionary variability in LRR residues of immune receptors can be quantified as computationally accessible Shannon entropy (Prigozhin and Krasileva 2021), and recent advances have illuminated functional diversity within closely related receptors (Lu et al. 2016; Saur et al. 2019; Bauer et al. 2021). These insights provide the groundwork to integrate sequence evolution with functional diversity, positioning language models as powerful tools for further exploration, which may empower the rational engineering of plant immune receptors.

One common theme for the future is the need to generate, share, and utilize good underlying experimental data for more accurate hypothesis generation and model training. For antibody structure prediction, over 3,400 antibody structures and 550 million antibody sequences were used for training (Ruffolo et al. 2023). Building such models for PRR-ligand or NLR-effector interactions will require well-organized and accessible datasets.

Recent comparative genomic approaches have curated a naturally diverse PRR ligand dataset, much of which has been experimentally tested in Arabidopsis or tomato (Colaianni et al. 2021; Parys et al. 2021; Stevens et al. 2024). Similarly, comprehensive resources cataloging NLRs and detailing plant-pathogen interactions have been developed, including RefPlantNLR, ANNA, NLRscape, PlantNLRatlas, and PHI-base (Urban et al. 2020; Kourelis et al. 2021; Liu et al. 2021a; Li et al. 2023; Martin et al. 2023). However, we may need to broaden these databases to include diverse data types, such as predicted immune receptors, effectors or ligands, and their complex structures, along with quantitative and qualitative interaction data. These resources could facilitate generating models for predicting mutation impacts and support the design of functional assays to capture binding, recognition, and immune outcomes. Standardizing data types and reporting methods will be crucial to make these resources broadly usable and to support machine learning applications. In this new journey, our community stands ready to explore new frontiers, boldly going where no one has gone before in plant immunity.

Conclusions

The growth of the availability and quality of data at the DNA, RNA, and protein levels has provided insights into the diversity generation and maintenance of plant immune receptors and has begun to answer the long-standing question of how strictly innate immune systems cope with rapidly evolving pathogens. Natural diversity uncovered at each layer poses challenges and opportunities for receptor engineering. Further understanding of the mechanisms behind high nucleotide diversity, transcriptional regulation, and immune receptors' ligand binding and activation will not only lead to new engineering strategies but also aid in continued development of computational predictive and generative models.

We posit that there is sufficient evidence to suggest that plant immune systems fit an anticipatory model of immune evolution, with a high likelihood of individual mutation maintained by population level-selection. The expression of immune receptors exists along multiple axes of regulation and is increasingly relevant to functional receptor prioritization and optimized control in engineering efforts. The new hope of protein structure prediction has revolutionized immune receptor design, but poses new opportunities and challenges for ensuring accurate and relevant structures. As we gain additional high throughput datasets through community-coordinated efforts, we can better leverage emerging machine learning approaches. Along with progress in understanding plant disease resistance, we face regulatory challenges, in particular which bioengineering efforts will be allowed in scalable agricultural settings and when they will be accepted by the general public. "Difficult to see. Always in motion is the future"—it is not yet clear how far we can apply synthetic immune receptors in crops. However, we must do our best to communicate that plants are natural engineers, and we have learned and continue to learn from them in our efforts.

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Author contributions

C.A.S. led conceptualization of the manuscript and drafting of the DNA diversity and contributed to expression variation. D.M.S. added PRR content across each section, led figure generation, and contributed to the immune receptor engineering section. W.W. led expression variation and its regulation. K.S. led immune receptor engineering. K.V.K. wrote the introduction, contributed to conceptualization of the manuscript, and contributed to the DNA section. All authors were involved in reviewing and editing.

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Supplementary data

The following materials are available in the online version of this article.

Supplementary Table S1. Summary of modeling confidence scores of receptors and their known ligands using different versions of Alphafold. PRR-Prediction holds the pTM and ipTM scores from Alphafold3 across several receptor-epitope combinations. NLR-Prediction holds the pTM and ipTM scores for predicted LRR/ID-effector complexes obtained from different versions and configurations of AlphaFold. DockQ socres were obtained by comparing AlphaFold models to experimentally determined structures. Sr50-AvrSr50 holds the ipTM scores of modeled Sr50 and AvrSr50 variants.

Supplemental Data Description. Description of data. AlphaFold_Models holds the raw outputs from all Alphafold modeling, which are also hosted on Zenodo (https://doi.org/10.5281/zenodo.14207050).

Conflict of interest statement. None declared.

Data availability

All raw data from Fig. 3 is available on Zenodo (doi:10.5281/zenodo.14207051) and in the associated supplementary information.

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