EVO-MPMI: From fundamental science to practical applications

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Abstract
In the unending coevolutionary dance between plants and microbes, each player impacts the evolution of the other. This article provides an overview of the burgeoning field of Evolutionary Molecular Plant-Microbe Interactions (EVO-MPMI), tracing its progression from foundational science to practical implementation. It delves into central concepts and methodologies, and offers a snapshot of current research. Moreover, the review sheds light on the practical applications of EVO-MPMI, particularly within the realm of disease control. Looking ahead, we discuss potential future trajectories for EVO-MPMI research, spotlighting the innovative tools and technologies propelling the discipline forward.

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Une valse a mille temps: The coevolutionary waltz between plants and microbes
While plant hosts are known to shape the evolution of symbionts and pathogens by driving changes within microbial genomes, the microbes leave their own mark on the evolution of their plant hosts. This has resulted in specific genetic signatures in microbial and plant genomes that are associated with past and ongoing interactions. These footprints provide evidence of the dynamic and reciprocal nature of plant-microbe coevolution, and can provide new insights into the underlying molecular mechanisms that drive these interactions. This is the basis of the emerging field of evolutionary molecular plant-microbe interactions (EVO-MPMI). By studying the mechanisms of plant-microbe interactions in the context of their evolutionary history, researchers can gain new insights into the complex and dynamic processes that shape the diversity and adaptability of these organisms. These coevolutionary systems are exceptional in that they can be studied over thousands of tempos, from millions of years to very short timescales, revealing anything from macroevolutionary patterns to rapid evolutionary adaptations [1-2].

Here we survey the EVO-MPMI field, covering recent developments from fundamental science to practical applications. The article explores key concepts and methods, and current state of research. It will also highlight the practical applications of EVO-MPMI in the areas of disease control. In addition, we discuss the future directions of EVO-MPMI research, including the emerging tools and technologies that are driving the field forward.

Beyond model species: The hidden diversity of molecular mechanisms
Our understanding of molecular mechanisms of plant-microbe interactions tends to be limited to a few model species and does not do justice to the huge biodiversity of interacting organisms. There is a whole world of microbial and plant diversity out there but not all of it is as accessible as some model species. Investigating molecular mechanisms in understudied,

and experimentally challenging systems has revealed novel pathways and unexpected innovations. For example, the recently compiled RefPlantNLR database underscores that, out of 59 orders of flowering plants, nucleotide-binding leucine-rich repeat (NLR) immune receptors have been experimentally validated in only 11 orders [3].

Recent years have seen a surge in research in non-flowering and non-vascular plants. *Marchantia polymorpha* was particularly quickly adopted as a popular experimental system [4]. Its mostly streamlined genome without whole genome duplications allows to test functional conservation of genes across large evolutionary distances. Like all plants the *Marchantia* genome encodes for conserved and lineage specific molecular mechanisms. Conserved biochemical defenses underpin *Marchantia* responses to oomycete infection but the purple pigment which accumulates at infection sites is not an anthocyanin, but chemically different and liverwort lineage-specific auronidins [5-6]. Bryophyte genome sequencing also uncovered new types of NLR genes with uncommon N-termini coding for putative hydrolases and kinases (for a recent overview see [7]). If truly lineage specific, these domains might aid in the engineering of crop immune receptors against angiosperm specific pathogens which might not have evolved mechanisms to inhibit them.

Diversity in plant-microbe interactions is also evident in nitrogen-fixing root nodules. Plant-secreted nodule-specific cysteine-rich (NCR) peptides probably evolved from defensin-like genes and regulate the symbiosis of nitrogen-fixing rhizobacteria by controlling the terminal differentiation of the microsymbiont (for a review see [8]). NCR peptides have evolved independently in two clades of legumes: the Inverted-repeat-lacking (IRLC, eg. *Medicago truncatula*) and the Dalbergoid clade (eg. *Aeschynomene indica*). Other legumes, such as soybean and *Lotus japonicus*, do not have NCR peptides. Likely, other mechanisms control the microsymbiont in these plants. Recent surprising results revealed that the nodule restricted expression of NCRs is not controlled by a lineage-specific mechanism. Instead, acquisition of *NCR* genes also involved recruitment of nodule-specific promoters which are regulated by existing transcription factors [9]. Similar to defensins, NCR peptides have antimicrobial activity and can terminate bacterial cell divisions with promising leads as antibiotics [8].

Functional effector studies based on the expression of effectors directly in plants have significantly added to our understanding of their biology. For Phytoplasmas, bacteria of the class of Mollicutes which reside within plant phloem cells and are transmitted by sap-sucking insects this was a real game changer. Phytoplasma effector expression in plants has led to the same morphological phenotypes observed during phytoplasma infections [10-12]. Then, the identification of host targets of these effectors was a key step in understanding underlying mechanisms such as SAP54's ability to degrade transcription factors in an ubiquitination-independent manner [12]. Mechanisms like these have wide potential for application beyond plants.

Recently, AlphaFold2 and similar deep-learning algorithms have enabled structure-model based comparisons in pathogens as well as genetically challenging arbuscular mycorrhiza fungi [13-14] and led to the identification of structurally similar effector candidates that are not conserved at the primary sequence level [15]. Facing this wealth of candidate effectors we now need to understand the molecular mechanisms underpinning their functions [16]. This requires the development of new assays and readouts beyond testing suppression of immune pathways and assessing their subcellular distribution *in planta*. Knowing how structure confers

function is also a starting point for protein engineering towards cell process control in disease and developmental contexts.

Conserved genes enable a wider perspective on structure and function

Comparative sequence analyses, conducted within a robust evolutionary framework, have emerged as a powerful approach to unravel molecular mechanisms and generate experimentally testable hypotheses. We illustrate this concept with examples centered on NLR class of plant immune receptors (**Figure 1**).

Recent studies have employed a phylogenomic approach to investigate the molecular evolution of the NLR protein ZAR1 in flowering plants (angiosperms) [17-19]. These investigations unveiled over 100 ZAR1 orthologs spanning monocots, magnoliids, and eudicots, indicating the exceptionally conserved nature of ZAR1, tracing its origins back to the early angiosperm lineages in the Jurassic period, around 220 to 150 million years ago. ZAR1 is known for indirectly detecting bacterial pathogens through binding pseudokinase decoys of the RLCK family. Remarkably, Adachi et al. [19] demonstrated that ZAR1 has been partnering with RLCKs for more than 150 million years, as supported by functional reconstructions of ZAR1-RLCK pairs from distantly related plant species (see **Figure 1**). These RLCKs are found in gene clusters across diverse angiosperms and are believed to have evolved from receptor kinases (RKs) involved in the recognition of microbial patterns. These findings suggest the presence of RK pathways dedicated to immunity during the Jurassic period, and that these pathways were already targeted by pathogens. Notably, the conserved ZAR1 kinase binding interface within the leucine-rich repeats supports this model, highlighting the enduring significance of this interaction across ZAR1 orthologs.

A striking conserved feature of ZAR1 is the MADA α1 helix, which plays a crucial role in defining the anion channel of the activated oligomeric resistosome [20-21]. This MADA motif is functionally conserved across a diverse range of angiosperms. In a recent study, Chia et al. [7] demonstrated that the MADA motif can be traced back to non-flowering plants, suggesting its origin to be at least 450 million years ago. Interestingly, in certain lineages of non-flowering plants, a variant of the MADA motif known as MAEPL defines the α1 helix of CC-NLRs (coiled-coil nucleotide-binding leucine-rich repeat receptors). Remarkably, this MAEPL variant is functionally interchangeable with the angiosperm MADA N-terminus [7]. However, whether the occurrence of the MAEPL variant is a consequence of historical contingency or represents an adaptive feature remains an intriguing question that requires further investigation.

This study exemplifies the value of integrating sequence conservation data with 3D structural analyses, as depicted in **Figure 1**. Looking ahead, as more protein structures become available, either through experimental efforts or computational predictions, the availability of ortholog series for a given protein will prove instrumental in uncovering conserved features and generating hypotheses regarding biochemical activities. Focusing solely on a single protein, such as the canonical Arabidopsis ZAR1, without tapping into the wealth of available sequence diversity, is unlikely to yield the same depth of insights. Additionally, variable features that deviate from the overall conserved sequences within an ortholog series can also provide valuable hypotheses. For instance, in eudicots, ZAR1 duplicated into the paralogous ZAR1-SUB proteins, which lack the kinase binding interface [17-19]. This observation led Adachi et al. [19] to postulate that ZAR1-SUB proteins may have neo-functionalized to bind

ligands other than RLCKs, offering a captivating avenue for further exploration and investigation.

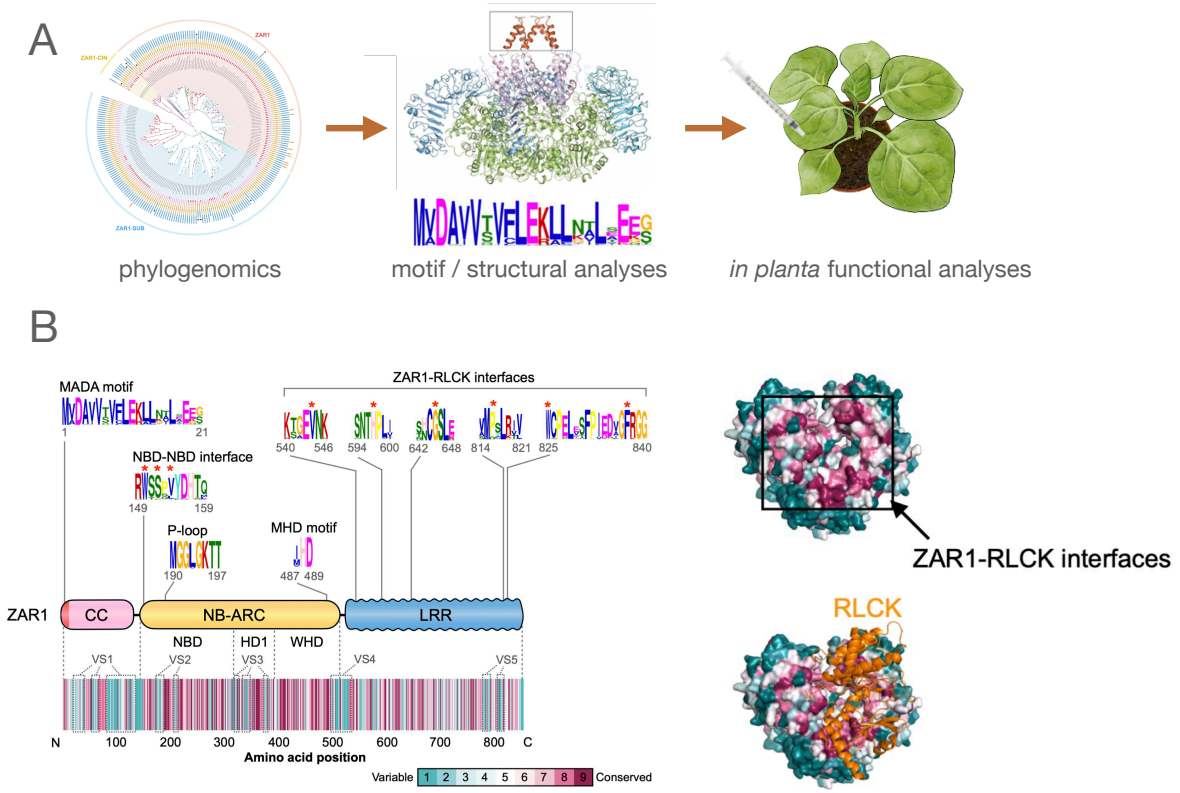

Figure 1. Conserved genes: A gateway to molecular mechanisms.

A. Workflow to identify conserved protein motifs and interfaces. This workflow adapted combines phylogenomics analysis, sequence conservation, and 3D structure comparisons. By generating experimentally testable hypotheses, this pipeline can help unveil novel biochemical features, offering insights into their functional significance. The figure is adapted from [19].

B. Conserved sequence patterns in the NLR immune receptor ZAR1 based on comparative analyses of an ortholog series. The schematic representation of ZAR1 protein highlights the position of these conserved sequence patterns across ZAR1 orthologs. Residues that have been functionally validated in Arabidopsis ZAR1 are marked with red asterisks. Motifs were identified using MEME and conservation scores calculated with ConSurf. The figure is adapted from Adachi et al. [17, 19].

Journeying through evolution: Ancestral reconstructions inform mechanistic models

Ancestral reconstructions of extinct proteins at pivotal phylogenetic nodes have gained popularity in EVO-MPMI studies [22-26] (**Figure 2**). These reconstructions play a crucial role in unraveling the evolutionary pathways taken by a protein, shedding light on evolutionary transitions and adaptive characteristics that provide valuable insights into molecular mechanisms. This approach allows researchers to reconstruct ancestral protein sequences that are no longer present in extant organisms. By tracing back evolutionary history, these reconstructions provide a glimpse into the ancestral forms of proteins, offering a deeper understanding of their functional and structural changes over time.

In a study on the neo-functionalization of a *Phytophthora infestans* protease inhibitor effector EPIC1 after a host jump, the ancestral gene was resurrected to investigate the hypothesis regarding shifts in its activity spectrum over evolutionary time [22]. The inhibition spectrum of the resurrected protein indicated that the ancestral protein had distinct activities from the derived protein from the sister species *Phytophthora mirabilis* consistent with the hypothesis that this effector has adapted to protease targets unique to its host plant *Mirabilis jalapa* [22].

Ancestral reconstructions enabled a plausible model of coevolution between APikL effectors from the blast fungus pathogen and host plants [23] (**Figure 2**). Ancestral reconstructions established the directionality of evolution and determined that the ancestral form of the APikL2 effector, D66 (GAT codon), preceded the derived form, N66 (AAT codon). Based on this insight, the D66N polymorphism is more likely to reflect an expansion in host-target binding rather than evasion of host immunity, and thus was proposed to be an adaptive feature that enabled binding to a new host target [23].

Ancestral reconstructions revealed the evolutionary history of rice immune receptors, Pik-1 and Pik-2, and their integrated HMA domain [24] (**Figure 2**). By reconstructing the evolutionary history of Pik-1 and Pik-2, coupled with functional studies, Bialas et al. [24] tested hypotheses regarding the adaptive evolution of the HMA domain. Employing ancestral sequence reconstruction, they demonstrated that two allelic variants of Pik-1 independently evolved from a weakly binding ancestral state to exhibit high-affinity binding to the blast fungus effector AVR-PikD. In another study, Snoeck et al. [25] used a combination of phylogenomics and ancestral reconstructions to show that INR, a cell-surface immune receptor that responds to inceptin molecules from caterpillars, emerged around 28 million years ago as a result of incremental mutations in the ancient form of the receptor [25].

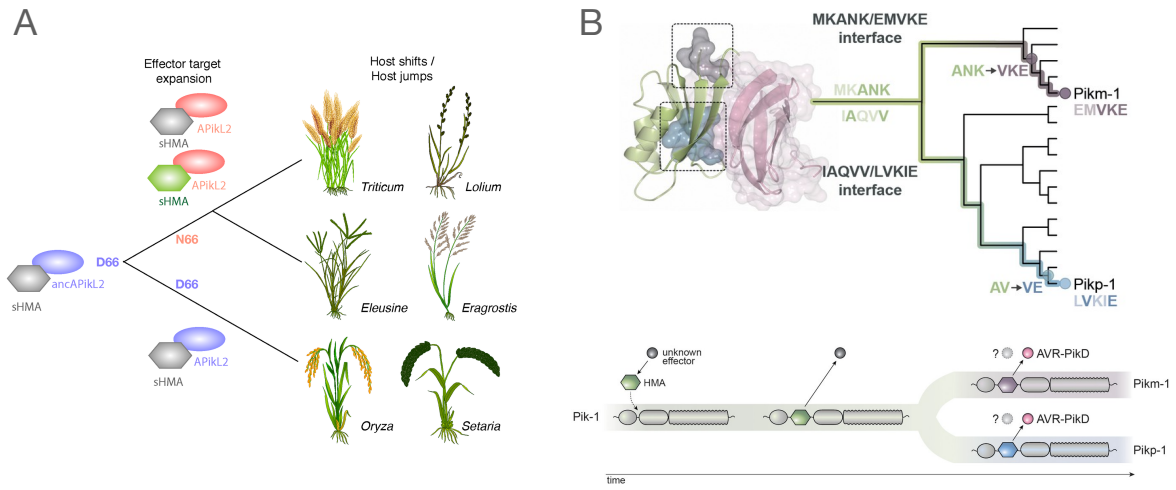

Figure 2. Ancestral reconstructions inform mechanistic models.

A. The Asp-66-Asn polymorphism in the APikL2 effector of the blast fungus *Magnaporthe oryzae* resulted in expanded binding to target small heavy metal-associated (sHMA) proteins. The figure depicts the host-specific lineages of *M. oryzae*, with annotation of the Asp-66/Asn-66 polymorphism (blue for Asp-66 and red for Asn-66). The model indicates that the Asn-66 polymorphism emerged prior to the differentiation of host-specialized lineages, and is likely to correspond to an expansion of the binding spectrum to sHMA proteins rather than evasion of detection by an immune receptor. The Figure is adapted from [23].

B. Molecular convergence of rice immune receptors Pikp-1 and Pikm-1 towards high affinity of the blast fungus effector AVR-PikD. Ancestral reconstructions revealed that the HMA domains of the Pikp-1 and Pikm-1 receptors

have undergone convergent evolution following distinct evolutionary paths. The Pikp-HMA domain evolved through AV-VE adaptations in the IAQVV/LVKIE region, while the Pikm-HMA domain acquired ANK-VKE mutations in the MKANK/EMVKE region. Bialas et al. [24] proposed a model in which the high-affinity binding of Pikp-1 and Pikm-1 allelic variants to the AVR-PikD effector has occurred relatively recently during the evolutionary history of this immune receptor. The Figure is adapted from [24].

Improbable destinies: Insights from convergent evolution

Convergent evolution occurs when different species or groups of organisms evolve similar adaptations to similar environmental cues [27]. Thus, studying examples of convergence can provide evidence for natural selection and adaptation and reveal advantageous traits.

An example are plant-pathogen effectors that converge on a limited number of host pathways, suggesting that there are critical processes for successful infection that pathogens have evolved to manipulate. Phylogenetically and structurally different effectors from unrelated microbial species target conserved signaling pathways and regulatory proteins important for host defense, such as immune signaling pathways [28-29], or the cellular endomembrane trafficking pathway [30]. Convergence can reveal the constraints and options underpinning a specific evolutionary process. Molecular studies are important to resolve whether effectors of divergent microbes target identical host proteins (Reviewed here: [31]) or different proteins within the same pathway or use the same molecular mechanism to do so. An example for the latter are bacterial and oomycete pathogens which independently have evolved effectors that act as substrate mimics for host 14-3-3 proteins [32-33]. Understanding the mechanisms by which effectors manipulate key pathways and processes provides opportunities for the development of new strategies for disease control.

Genes underpinning plant immunity also display signs of convergent evolution. Immune receptors underpin distinct elicitin recognition in related Solanaceous plants [34]. Barley and Arabidopsis have convergently evolved different nucleotide-binding leucine-rich repeat (NLR) proteins to detect effectors that structurally modify PBS1-like kinases [35].

Convergence also aids in reconstructing evolutionary history. Genome sequence analyses have shown the independent evolution of the ectomycorrhizal lifestyle of several independent fungal lineages from their saprotrophic ancestors. This included the convergent losses of different plant cell wall-degrading enzymes [36]. In the endomycorrhiza formed between most land plants and arbuscular mycorrhiza (AM) fungi, PHT1 type phosphate transporters play a key role in nutrient uptake into plant cells. While PT4/PT11 clade PHT1 phosphate transporters are transcriptionally upregulated in most studied angiosperm models, some Solanaceae and the bryophyte *Lunularia cruciata* upregulate PHT1-type transporters from non-PT4 clades, suggesting that the molecular mechanisms underpinning nutrient transfer may have evolved convergently [37-38].

Evolutionary tinkering paves the way for bioengineering

In the words of the eminent biologist Francois Jacob, evolution can be likened to a skilled tinkerer, subtly modifying biological molecules over the course of millions of years [39]. Biotechnologists have taken inspiration from this natural process, striving to replicate and expedite evolution within the confines of our laboratories. In the realm of EVO-MPMI, numerous examples demonstrate the transformative power of evolutionary reconstructions in guiding bioengineering endeavors.

The evolutionary trajectory of TAL effectors (TALEs), predominantly found in the bacterial pathogens and symbionts Xanthomonas and Burkholderia, has captivated researchers in the

field. These effectors have evolved the remarkable ability to bind DNA that is governed by a protein-to-DNA cipher that enables TALEs to bind specific DNA sequences [40-42] (**Figure 3**). The initial discovery of the cipher was based on comparisons of related and unrelated TALEs converging onto the same promoter elements. Subsequently, evolutionary studies have provided insights into how TALEs evolve to maintain binding to susceptibility gene promoters [43]. This knowledge has guided the design and engineering of synthetic TALE-derived DNA-binding proteins, which can be combined with other domains to enable gene editing and modulation of transcriptional activities, among many applications [44]. TALEs have also applications in plant disease resistance (**Figure 3**). Among these is the identification and design of decoy sequences that trap pathogen TALEs to activate executors of immunity [45] and the use of experimental evolution to predict TALE adaptations that evade binding to sequences that activate immunity [46].

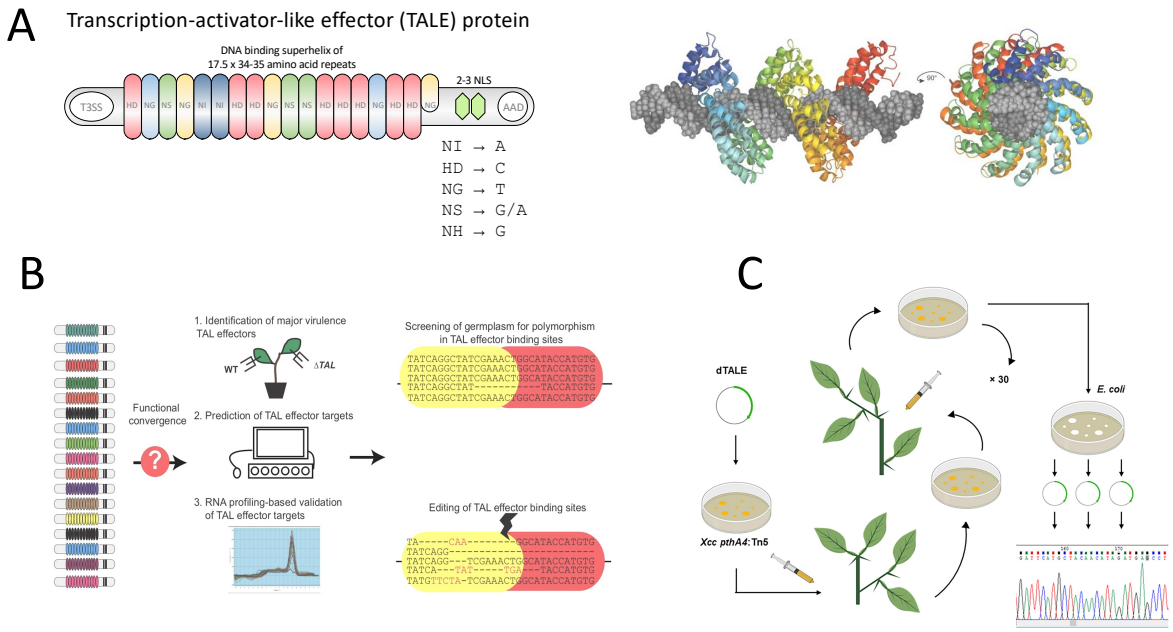

Figure 3. Evolutionary tinkering of TAL effectors guide bioengineering and disease resistance strategies.

A. Structure of TALE proteins. TALE proteins consists of a series of repeats, each containing two hypervariable amino acids that bind to DNA following a specific code. T3SS: Type III secretion signal, NLS: nuclear localisation signal, AAD: acidic transcription activation domain. 12/13th repeat-variable diresidues RVD are listed in each repeat. The code table lists common RVD-to-DNA base specificities.

B. Workflow for the identification and design of sequences that trap TALEs to activate executors of immunity. Adapted from [45].

C. Experimental evolution pipeline to predict TALEs adaptations that evade binding to sequences that activate immunity. Adapted from [46].

Another area where evolutionary reconstructions have been instrumental is in the realm of NLR-IDs (NLR immune receptors with integrated domains). This remarkable process—when an unconventional domain is integrated into a classical NLR architecture to bait pathogens—has occurred hundreds of times throughout plant NLR evolution [47-48]. By reconstructing the evolutionary history of the Pik NLR pair, which includes an NLR-ID [49-50], Bialas et al. [24] and De la Concepcion et al. [51] unlocked the bioengineering principles for building made-to-order NLR-ID proteins with enhanced functionalities. This work has set the stage for

pikobodies, an approach that seeks to mimic the evolutionary process by integrating animal antibody domains into NLR proteins [52]. If nature figures out how to bioengineer an NLR-ID every few millions of years, why can't we make this routine in our laboratories?

Evolutionary reconstructions have also helped produce a framework for how functionally linked immune receptors undergo co-adaptation to provide an effective and regulated immune response against pathogens [48, 51]. This has revealed, for instance, how mismatched Pik-1/Pik-2 pairs can lead to autoimmune or hypoactive phenotypes and which amino acids modulate these activities [51], helping to add to the toolbox for pikobody bioengineering [52-53].

Future prospects

The future of EVO-MPMI appears promising, with opportunities for both scientific advancements and practical applications. The development of innovative pipelines and methodologies will be crucial. Such advancements will enable comprehensive and efficient analysis of large-scale data, incorporating high-throughput sequencing, comparative genomics, and functional assays. By taking a holistic view of the complex interplay between plants and microbes, scientists will be able to interpret the underlying molecular mechanisms driving these interactions in the light of evolution.

One key avenue that is set to shape the field's trajectory is the integration of molecular evolution with structural biology (**Figure 1-2**). By combining these two powerful approaches, researchers can delve into the intricate mechanisms and adaptive features of plant-microbe interactions at the molecular level, unraveling their evolutionary dynamics and gaining deeper insights.

Nobel Prize laureate Frances H. Arnold's groundbreaking concept of "innovation by evolution" [54] serves as an inspiration for EVO-MPMI researchers. Drawing upon the power of natural selection, they can leverage evolutionary principles to drive practical applications. By identifying and optimizing proteins and molecules with desired functionalities, the field holds the potential for groundbreaking biotechnological applications.

Declaration of competing interest

S.S. holds patents on TALEs. S.K. receives funding from industry and has filed patents on NLR biology.

Data availability

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

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